Developing Molecular Methods to Identify and Quantify Ballast Water Organisms: A Test Case with Cnidarians

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List of Acronyms

DGGE - denaturing gradient gel electrophoresis
DMSO - dimethyl sulfoxide
DNA - deoxyribonucleic acid
ITS - internal transcribed spacer
mtDNA - mitochondrial DNA
PCR - polymerase chain reaction
rRNA - ribosomal
RNA - ribonucleic acid
RFLPs - restriction fragment length polymorphisms
SSCP - single strand conformation polymorphisms

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Executive Summary

Background

The potential for organisms to be transported by ballast water is well documented. Furthermore, biological invasions mediated by ballast water transport have led to some rather severe economic and ecological consequences as seen in the examples of the zebra mussel and green crab. Unfortunately, this is not necessarily a new phenomenon. As long as ships have traveled so have these sorts of stow away organisms. However, what has changed is the rate of invasions. Bigger ships, with greater volumes of ballast water, are traveling faster which increases the abundance and survival of organisms in their ballast tanks.

The growing recognition of the consequences of invasive species has led to much effort in improving our basic understanding of the role of ballast water. One key question is simply what species are being transported? Various studies have documented the presence of a tremendous diversity of non-indigenous species in ballast water. Importantly, the authors of these studies acknowledge that these numbers are underestimates since larval forms, as well as the adults, of species in many groups cannot be easily distinguished based on morphology. Thus, characterization of the taxa present in ballast water samples is often restricted to the taxonomic level of order, class or even phylum.

The goal of this project was to adapt standard molecular methods into a novel approach for quantifying the abundance and diversity of organisms in the ballast water of DoD vessels. The need for this work is based on the difficult and time-consuming task of using morphology alone to identify ballast water organisms. Furthermore, full identification of certain taxa is not always possible, leading to an underestimate of the diversity of organisms present. A similar problem is faced by microbiologists in determining the bacterial species present in environmental samples. Environmental microbiologists have turned to a molecular approach since these methods use the DNA of the organism, not its morphology, to make an identification. While molecular techniques have provided robust estimates of species diversity of mixed bacterial communities in soil and water, the potential of this approach to identify ballast water organisms has not been fully explored.

Given the exploratory nature of this project, we have chosen to focus on cnidarians as they are a difficult group to work with for a variety of reasons:

- cnidarians (especially their larval forms) often possess few morphological characters on which to base identifications;
- cnidarians are fragile and easily damaged during sampling; and
- cnidarians are often present only in small numbers making detection even more difficult.

A molecular approach has the potential to overcome many of the obstacles encountered in using traditional identification methods, and we hope to demonstrate this with a rather difficult group of organisms. Should the protocol pass this rather stringent test case, then it should be straightforward to apply this methodology to other groups of organisms common in ballast water (*e.g.* annelids, crustaceans and mollusks). An additional benefit of accurate species identifications is that this information might be suitable for determining if mid-ocean ballast water exchanges have taken place. The relative proportion of near-shore and offshore species can be used as an indicator of the ballast water source.

Methods

All of the molecular methods employed are standard techniques. Below we briefly describe the methods associated with each of the three phases of development and evaluation.

Phase I. Characterize the molecular markers needed for identifications

The objective was to determine which portion(s) of the genome would provide the appropriate level of taxonomic resolution.

- Specimen sampling. The first step was to obtain representative samples of various cnidarian species to provide working material for our search for appropriate markers. The exploratory nature of this research dictated a broad, but not necessarily exhaustive, sampling of species. We obtained representatives from each class of cnidarians (especially Anthozoa, Hydrozoa and Scyphozoa) from both the northern portion of the Atlantic as well as the Gulf of Mexico.
- Molecular marker development. We initially focused on the 18S rRNA gene since a considerable amount of sequence data was already available for a variety of cnidarian species. In addition, we examined the usefulness of the internal transcribed spacer (ITS) region of the rDNA gene group and the mitochondrial 16S rRNA gene. To evaluate these genes as potential markers, we acquired preliminary sequence data from a variety of cnidarians either through published data or our own lab work. These data then allowed us to evaluate whether a given gene possessed regions that were sufficiently conserved or variable enough to discriminate among the different taxa.

Phase II. Laboratory evaluation of markers - identification and quantification

This objective was to determine if our protocol would be able to detect the presence of cnidarians in prepared samples of a mixture of DNA from a variety of organisms. In addition, by using a dilution series of templates at known concentration, we evaluated the lower detection limits of this protocol. These data would also be valuable in any attempt to quantify the relative abundance of each taxa in a sample.

Phase III. Evaluate markers with ballast water samples

We tested our protocol on one set of ballast water samples provided by Dr. Eric Holm of the Naval Surface Warfare Center. In addition to the ballast water samples we used environmental plankton tows from Mississippi Sound as mock ballast water samples. Since we were not certain if either the ballast water or environmental samples would actually contain cnidarians, we tested the ability of our methods to detect single individuals of planktonic cnidarians by using individual *Hydra* as a surrogates in our protocol.

Results

Phase I. Characterize the molecular markers needed for identifications

We were able to obtain a total of 26 species consisting of 10 anthozoans, 9 hydrozoans, 6 scyphozoan and 1 cubozoan. Most of the data we used in this part of our protocol design was already available on GenBank, but these samples were used throughout laboratory testing phases.

After analysis of the sequence alignments both within and between taxonomic groups, we rejected 18S rRNA as a marker for use in this study. This gene lack the right mix of conserved and variable regions that would enable us to design robust taxa-specific primers. Examination of the mitochondrial 16S rRNA sequences indicated that it would be useful as a marker for our protocol. We were able to identify conserved regions that differed among in the group, which provided us a location to the design diagnostic primers. Also, the variability within each group

seemed sufficient distinguish among species or at least genera. After the identification of the 16S rRNA gene as an appropriate marker, we did not pursue any additional work on ITS. However, the high degree of within group variation, would seem to indicate that it would make a useful secondary marker in obtaining accurate species identifications.

We designed eight primers for 16S rRNA that were intended to specifically amplify major taxonomic groupings of cnidarians Unfortunately, the taxa-specific primers were not all equally effective. The hydrozoan and scyphozoan primers tended to amplify anthozoan groups. To get around this problem sequentially tested samples - first with anthozoan primers, and then any samples not identified as such were tested with the hydrozoan and scyphozoan primers. Once identified to this level, RFLPs were used to identify taxa within each major taxonomic grouping to the level of genus or species. Depending on the group, these diagnoses required the use of either a single enzyme to several enzymes in combination. We found that RFLPs was a robust means of identification in the Hydrozoa and Scyphozoa, but that there was a lack of complete taxonomic resolution among the Anthozoan species. This is not a major problem, as the RFLPs still seem robust at least to the level of family and genus. Should a more refined estimate of the numbers of species be required other techniques such as single-strand conformation polymorphisms (SSCPs) and denaturing gradient gel electrophoresis (DGGE) might be employed.

Phase II. Laboratory evaluation of markers - identification and quantification

We were able to clone PCR fragments produced from a mixed pool of DNA from a variety of cnidarian species. As we characterized increasing numbers of clones from this experiment we found that the total number of species recovered increased relatively rapidly as more clones were sampled. In an attempt to evaluate the lower detection limits of the PCR, we compared the ability of two brands of *Taq* polymerase to amplify DNA at a variety of combinations. We found that with one brand we were able to obtain strong amplifications even down to the level of 250 pg of DNA. Lastly we determined that our primers would not amplify other taxonomic groups (a crustancean and polychaete) that might commonly be present. Similarly, we demonstrated that the PCR was capable of selective amplification even in a mixed sample of cnidarian and non-cnidarians.

Phase III. Evaluate markers with ballast water samples

The ballast tank samples we received appeared to have very few individuals of zooplankton and no DNA was detected by the agarose gel check of extractions. In the environmental plankton tows there was abundant life, although most of it appeared to be phytoplankton, and we were able to obtain high molecular weight DNA. Likewise, the DNA extractions of individual *Hydra* were successful. Attempts to amplify the DNA extractions from the ballast water samples were not successful, but the environmental plankton tow samples also did not produce any amplifications. However, since the individual *Hydra* produced robust amplifications this might suggest that cnidarians were absent in the ballast tank and plankton tow samples. We also demonstrated that this lack of amplification was not due to the presence of PCR inhibitors or nontarget DNA in these samples.

Conclusions

While the developmental process and lab tests were quite promising, as of yet we have been less successful in carrying these techniques from the lab over to actual field samples. However, the fact that we were successful in our work with surrogates of small planktonic cnidarians (the *Hydra*) leads us to believe that it would be premature to give on the protocol just yet. Amplifications of extractions of single *Hydra* as well as mixtures of *Hydra* and plankton sample DNA were all successful. This suggests to us that had cnidarians been present in the ballast tank and plankton tow samples that they would have been detected by our protocol.

This project has been successful in taking the first step in bridging the gap between the potential and the application of molecular techniques. Yet, there are several follow on efforts that could been undertaken to better develop the protocol presented in this report. First and foremost would be to obtain additional ballast tank samples, with a larger number of taxa present. As the field validation of our protocol is the only accomplishment that we lack, we intend to work up additional samples. Based the robustness of our lab tests we remain confident that our protocol will be successful for its intended purpose providing we test it under the appropriate conditions. The second follow up effort would be to refine our ability to distinguish among the taxa using our marker. We had initially focused on a RFLP approach because it is technically simple and relatively inexpensive. However, we recognize that is does not have the discriminatory ability that other techniques posses. If the additional tests of field samples prove promising, then it would be useful to explore other methods and potentially add the to our protocol.

As far as the economic feasibility of our protocol, we can provide an estimate with regard to the cost of reagents and time, but the total cost would depend on how many samples would need to be processed from a single vessel. The overall cost in reagents should be roughly under $200, and the time required to process a sample from start to finish would be about eight days for a single technician. However, each task would not necessarily consume the technician's time for the entire day so multiple samples could be in the work flow simultaneously. An assessment of the attractiveness of this technology must be based on the need/desire for a certain level of taxonomic resolution in identifications. At the grossest taxonomic level, visual identification are certainly the best and easiest. For example, if you simply wanted to know the relative numbers of crustaceans vs. mollusks. But beyond the level of family in many cases, the molecular approach would probably win out due to the high taxonomic skill level required by the technician as well as the robustness of taxonomic identifications a molecular approach would provide.

One additional application of this protocol that will warrant examination is its use in identifying hull fouling organisms. Once again taxonomic designations for these organisms may be difficult, especially if the specimens are damaged in the act of removing them. However, as long as they are preserved appropriately, this protocol can be used to identify them based on their DNA.

Transition Plan

The information about the protocol will be conveyed to the scientific community in a peer-reviewed publication. By getting the concept and methodology of our protocol out into the

literature we hope that we can stimulate interest and promote evaluation by other researchers who will be able to test and validate the feasibility and rigor of the methodology for themselves. This process will certainly lead to improvements in the protocol when it is applied to novel situations beyond the ones we devised for testing in the developmental stages.

While the cost assessment that we have provided is rather rough, we will point out that the deliverable aspect of this project (a means to identify cnidarians in ballast water) is a product that is not realistically obtained by any other method. Another deliverable aspect of this project will be the demonstration of a protocol that, given suitable development, can be applied towards the identification of any of the groups of organisms commonly found in ballast water or in a hull fouling community.

Recommendations

Since this project was designed to demonstrate a proof of concept, we are not necessarily at the stage where our deliverable product can be immediately transitioned into an operational format. However, with some additional testing of actual ballast samples under a variety of conditions we will be in a position to better evaluate the future of our protocol. Similarly, the basic methodology, once in the literature, may subsequently be developed by others for other taxonomic groups that may be of special interest or relevance.

Objective

The objective of this exploratory research is to describe molecular markers and refine the methods necessary to identify cnidarian taxa in ballast water samples. All the molecular methods to be employed are standard techniques. The novel aspect of this work that will require experimentation and optimization is developing protocols that apply these techniques to detect, identify and quantify ballast water organisms.

This project can be divided into three phases, each with certain methodological challenges. We do not consider these issues to be insurmountable, but we mention them now to make it clear that we are aware of what will be required to develop a functional and feasible molecular protocol. The specific goals of this project are detailed for each phase.

Phase I. Characterize the molecular markers needed for identifications.

The objective is to determine which portion(s) of the genome will provide the appropriate level of taxonomic resolution. Complete taxa identifications may require using a range of markers in a hierarchical fashion. For example, one gene might be used to identify the presence of cnidarians to the level of class or family, while another gene would be employed to refine the identification to the level of genus or species. The strength of a hierarchical approach is that you can choose the taxonomic level at which you are seeking to identify presence or absence. In other words, the taxonomic diversity of cnidarians in a ballast water sample could be assessed anywhere from the level of class down to a precise enumeration of the species present.

Phase II. Laboratory evaluation of markers - identification and quantification.

The objective is to determine if our protocol will be able to detect the presence of cnidarians (via their DNA) in prepared samples containing DNA from other organisms. In addition, by using a dilution series of templates at known concentration we will evaluate the lower detection limits of the molecular protocol. These data will be valuable in quantifying the relative abundance of each taxa in a sample.

Phase III. Evaluate markers with ballast water samples.

The objective will be to perform the real test of the methods developed. The performance of the markers should be predictable based on tests from the first two phases of the project. However, we still need to demonstrate that the methodology is applicable outside of the controlled conditions in the lab.

Background

The Problem and Approach

The fact that organisms are transported by ballast water is well documented (*e.g.* Carlton and Geller 1993; Ruiz *et al.* 1997; Smith *et al.* 1999). The tremendous number and diversity of non-indigenous species present in ballast water is also well documented. Carlton and Geller (1993) reported that a minimum of 367 different taxa representing all major and many minor phyla were present in ballast water samples taken from tankers in Coos Bay, Oregon. Importantly, they acknowledged that this number represented an underestimate since many species are morphologically indistinguishable. Larval forms, as well as the adults, of species in many groups cannot be distinguished based on morphology. Thus, characterization of the taxa present in ballast water samples is often restricted to the taxonomic level of order, class or even phylum. Therein lies a major obstacle to a comprehensive understanding of the role of ballast water in the spread of non-indigenous species. We lack the ability to know exactly which species are being transported, released and potentially becoming established in an area.

The standard method of identifying ballast water organisms is via their morphology using light microscopy. Three limitations are inherent to this method.

- Accurate identifications require considerable taxonomic knowledge by the technician.
- As mentioned above, the lowest taxonomic level to which an organism can be identified is often limited by the fact that the larval forms of many species are essentially indistinguishable. In order to obtain an identification for some organisms it is necessary to culture the unknown until it develops into a more advanced larval stage.
- In order to culture unknown organisms, the organisms must be kept alive, which requires a rapid turn around from the time of sampling to placing them into a culture environment.

The problem at hand with a ballast water sample is analogous to the problem faced by environmental microbiologists. How can you characterize the species composition of a mixed community from an environmental sample (*e.g.* water or soil)? The analogy is especially apt since microbiologists are also unable to identify many bacterial species given a lack of diagnostic morphological characters. The solution to this problem is not to examine the morphology of the organism but to examine their DNA (*e.g.* Pace 1997). The standard approach to species identifications of bacteria from an environmental sample is to first perform a DNA extraction of the sample. This process yields a mixture of DNA from all organisms (bacteria and others) present in the sample. From this bulk DNA sample the polymerase chain reaction (PCR) is used to selectively amplify a portion of the bacterial genome. If you use the appropriate primers, copies of the target gene will be made for each species present. The amplified DNA fragments are then cloned. Cloning is a method by which individual DNA fragments are incorporated into a circular piece of DNA (a plasmid), which is then inserted into a bacterium (*E. coli*). Culturing the transformed bacteria allows them to reproduce, which at the same time increases the copy number of the cloned DNA fragment. The purpose of the cloning is to isolate one piece of DNA from the pool of copies produced by the PCR. The isolated fragment corresponds to one of the species of bacteria present in your environmental sample. The species identity is then determined, typically by sequencing the cloned gene and then comparing it to sequences from known species.

A powerful aspect of PCR is the ability to selectively amplify a specific gene from a specific set of taxa. This is a feature used by microbiologists to selectively amplify genes from only the bacteria present in the sample. Similarly, Borneman and Hartin (2000) have used specific primers to only amplify the target gene from the four major phyla of fungi from environmental samples. However, the selective ability of PCR may be taken even further by using primers specific for certain types of bacteria. For example, Michotey et al. (2000) were able to identify denitrifying bacteria in marine samples, and Bernhard and Field (2000) detected anaerobic fecal bacteria in water samples.

We are not implying that molecular techniques have never been applied in the study of marine or ballast water organisms. For example, using molecular techniques to identify larval organisms is not a new endeavor. Two studies (Bilodeau *et al*. 1999; MaKinster *et al*. 1999) reported using middle repetitive elements in the genomes of decapod crabs as a marker to identify larvae. Bilodeau et al. (1999) were able to identify a single larva of *Sesarma reticulatum* in a plankton sample, while MaKinster et al. (1999) were able to detect single larvae of *Menippe adina* and *M. mercenaria*. In a more recent example, Deagle et al. (2003) present a PCR-based test of mitochondrial DNA for *Asterias* (an echinoderm) larvae in ballast water samples. However, this technique, for a variety of reasons, is only useful for identifying one or two species at a time. J.B. Geller (pers. comm.; Moss Landing Marine Lab) has used the PCR, clone and sequence approach on ballast water samples. He was able to estimate total species diversity based on the number of unique sequences using this method, but he did not attempt to provide a taxonomic designation for each sequence. The methods that we are proposing to develop will both quantify the diversity of species as well as provide an identification (described in detail below). Full implementation of this approach requires knowledge of the sequences for various genes for the taxa present in ballast water samples. Fortunately, a great deal of background data for many invertebrate groups is already available on GenBank (the National Center for Biotechnology Information's sequence database; http://www.ncbi.nlm.nih.gov).

Why cnidarians?

Cnidarians are not the most abundant organisms in ballast water samples (crustaceans are; *e.g.* Smith *et al.* 1999), but there are other reasons for using cnidarians as a model group to develop the molecular protocol. Cnidarians are fragile and may suffer damage during the sampling process. While this may hinder morphological identifications, this will not be a problem for molecular methods. The DNA from the sample is the unit being examined, not the organism. In most studies, even intact cnidarians have not usually been identified beyond the level of class. Molecular methods will provide a way to refine identifications. Lastly, invasive cnidarians have demonstrated that they can become serious problems. During the past summer, the Australian jellyfish, *Phyllorhiza punctata*, exploded in numbers throughout the northern Gulf of Mexico posing a potential threat to local fisheries (Larsen et al. 2001).

Indicators of ballast water exchange

Inshore and offshore communities are potentially quite different in terms of species composition, and thus the species composition of a ballast water sample should reflect its source. This provides a means by which the taxonomic designations obtained via the molecular protocol can be used to assess whether ballast water exchange occurred. Shifts in cnidarian species composition from inshore to offshore environments should prove to be useful indicator species.

For example, species in the class Scyphozoa (*e.g.* jellyfish) tend to be mostly pelagic and should be highly represented in the offshore community. Other species, such as in the class Anthozoa (*e.g.* corals) and certain orders of Hydrozoa (*e.g.* hydroids), tend to be sessile and would be most common closer to shore. Admittedly, larval stages in these groups may be widely dispersed, but examination of the overall composition and diversity of species present should prove to be a reliable indicator of the source of a ballast water sample.

Materials and Methods

The ultimate objective of this project was to develop a molecular approach of identifying cnidarian species from a ballast water sample. This process involved several steps. First, we needed to identify an appropriate molecular marker. Then it was necessary to develop the methods necessary to selectively detect and identify cnidarian species. This approach was then tested in the lab in order to assess its detection ability and limits. Lastly, the protocol was tested on actual ballast water samples.

Phase I. Characterize the molecular markers needed for identifications.

Specimens

Although a tremendous amount of data is available on GenBank, specimens representing the various cnidarian groups were still required for testing the protocols developed. These specimens were acquired through personal collections, from colleagues or from commercial suppliers (Carolina Biological Supply Co. and Marine Biological Laboratory). Specimens from commercial suppliers were shipped alive to the lab where they were immediately processed. Other collections were made by preserving a tissue sample in a 100% ethanol or a salt saturated solution of DMSO and EDTA (Seutin et al., 1991). Small specimens were preserved whole, while a section of dense tissue such as tentacles or gonads was taken for larger specimens.

DNA Isolation

Total genomic DNA was extracted from the fresh or preserved tissue samples using one of three procedures: the protocol of Geller and Walton (2001), the DNAzol extraction method (Chomczynski et al. 1997) or the QIAamp Tissue Kit (QIAGEN Inc., Valencia, CA). The quality of the extracted DNA was examined by gel electrophoresis on 1% agarose gels, stained with ethidium bromide (0.5 μg/ml) and then visualized under ultraviolet light. DNA concentrations were measured by a Hitachi F-2000 fluorescent spectrophotometer.

Marker Identification

The ideal molecular marker would be a gene that was conserved in some areas across the different cnidarian groups, but highly variable in other regions within these groups. The conserved regions would allow the design of primers for the selective amplification of specific cnidarian taxa. The variable regions would provide the diagnostic markers to identify different cnidarian species within these groups. However, for the marker to be useful there needs to be differences among species but limited variation within a species that might confuse taxa identifications. We identified three regions as candidates either alone or in combination to serve as our molecular marker. These genes were 18S rRNA (nuclear), 16S rRNA (mitochondrial) and ITS (internal transcribed spacer region of the nuclear ribosomal gene complex).

We chose to first examine 18S rRNA as it has been the molecule of choice for many molecular systematic studies within and among the major groups of cnidarians (e.g. Bernston et al. 1999, 2001; Collins 2002). Thus, a tremendous amount of data was already available to use in this study. Animal mitochondrial DNA is also commonly used in systematics as well as phylogeographic and population genetic studies. A variety of systematic and evolutionary studies have employed 16S rRNA (Cunningham and Buss 1993; France et al. 1996; Romano and Palumbi 1996). However, there seems to be range in the extent that mtDNA varies within a

species. McFadden et al. (2000) found limited amounts of intraspecific variation in anthozoans, while the scyphozoan *Aurelia* has demonstrated considerable variation within species and among congeners (Dawson and Jacobs 2001; Schroth et al. 2002). However, overall, the evidence to date indicates that there is a much slower rate of evolution in cnidarian mtDNA compared to other groups. In particular, anthozoans show a 10-20 fold slower rate and have limited amounts of intraspecific variation (Shearer et al. 2002). Other cnidarian groups also demonstrate a slower rate of evolution, but at least in the case of *Aurelia* intraspecific variation is present. While this has implications for the usefulness of mtDNA at the intraspecific level, it seems that this would be a beneficial feature for the use of mtDNA intended in this study. The internal transcribed spacer region (ITS) has been found to be highly variable. And for this reason, given the dearth of mtDNA intraspecific variation within cnidarians, it has seen use in both phylogeographic (Rodriguez-Lanetty and Hoegh-Guldberg 2002; Schroth et al. 2002) and genus level systematic studies (Dawson and Jacobs 2001; Diekmann et al. 2001).

18S rRNA.— Sequences were obtained from GenBank while others were obtained from specimens on hand. We used the universal 18SF primers (18SF and 18SR) of Medlin et al. (1988) as modified by Bernston et al. (1999) to amplify the complete 18S rRNA. Amplifications were conducted in a total volume of 50 µl using 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 0.01% gelatin, 2 mM $MgCl_2$, 200 µM dNTPs, 1.5 units *Taq* polymerase, 0.3 µM of each primer, approximately 100 ng template DNA and water to the final volume. PCR cycling conditions consisted of an initial denaturing step of 95°C for 1 min followed by 30 cycles of 1 min at 95°C, 1 min at 50°C and 1 min at 72°C. A final elongation step of 7 min at 72°C ended the cycle. PCR products were gel checked on 1% agarose gel stained with ethidium bromide and then cleaned prior to sequencing using the QIAquick PCR purification kit (QIAGEN Inc., Valencia, CA).

Sequencing was conducted using the 18SF and 18SR primers as well as the internal primers 373F and 1200R of Weekers et al. (1994). Reactions were conducted using a BigDye Terminator cycle sequencing kit (Applied Biosystems, Foster City, CA) and cleaned with Centri-Sep columns (Princeton Separations, Adelphia, NJ). Gel runs were performed at the Iowa State University DNA Sequencing and Synthesis Facility. The sequence data were aligned and edited with Sequencher 4.1 (Gene Codes Co., Ann Arbor, Michigan). Data from GenBank was also aligned using this program.

The suitability of the sequence as a marker was assessed in several ways. First, we examined the alignment in an attempt to identify conserved regions within taxonomic groups where we could place primers for selective amplifications. These potential primer sites were identified by eye and the suitability of the primers was tested using the Oligo toolkit of Operon (http://oligos.qiagen.com/oligos/toolkit.php). Two other approaches were used to obtain species levels identifications within groups. The first was to examine the sequence for potential restriction site differences between species using Sequencher 4.1. In addition, we also examined the restriction fragments for potentially diagnostic size variation between species.

16S rRNA.— Again, sequences were obtained from GenBank and others were obtained from specimens on hand. A variety of primers were used in the amplifications beginning with the universal primers of Palumbi et al. (1991) as modified by Cunningham and Buss (1993). We

also tested the 16S L5' and H5' of Schroth et al. (2002), and we eventually used preliminary sequence data to modify the 16S H5' primer to better match the various cnidarian groups. All PCR, sequencing and analyses of data were performed as described for the 18S rRNA. However, in the initial PCR trials annealing temperatures anywhere from 40-55°C were used.

ITS.— Again, sequences were obtained from GenBank and others were obtained from specimens on hand. We began our attempts to amplify the ITS region with the universal primers ITS-4 and ITS-5 of White et al. (1990) and the primers ITS F5' and ITS R5' of Schroth et al. (2002). We later replaced the ITS-4 primer with a new primer (28S-R2) set in the conserved region of the flanking 28S rRNA gene. All PCR, sequencing and analyses of data were performed as described for the 18S rRNA.

Taxa identifications

As described above under (Marker Identification), we designed primers that would selectively amplify specific groups of cnidarians. The identification of individuals beyond the major taxonomic groupings was accomplished via the analysis of restriction fragment length polymorphisms (RFLPs). Restriction enzymes cut DNA at specific recognition sites so genetic variation between sequences can be determined by examining the presence/absence of cut sites. Diagnostic restriction enzymes were selected by comparing the RFLP profiles of species within each taxonomic group using Sequencher 4.1. Aliquots of the amplified marker gene were then aliquots were treated with one of each of the diagnostic restriction enzymes following the manufacturer's recommendations (New England Biolabs, Beverly, MA). Each digestion reaction was conducted in a 20µL volume with 10µL of the PCR amplification, 1X of the appropriate buffer, 0.2µL of the restriction enzyme, and water to the final volume. Digestion reactions were incubated at 37°C for 4-6 hrs. The fragments produced by these digests were separated on 2% agarose gels, stained with ethidium bromide (0.5 µg/ml) and then visualized under ultraviolet light. Each band produced by a restriction digest was scored for size by comparison to known size standards (Promega 1kb DNA ladder and 100bp DNA ladder)

Phase II. Laboratory evaluation of markers - identification and quantification.

Detection ability

When starting with a bulk DNA extraction, PCR should result in a pool of products amplified from whatever species are present in the sample. Once these PCR products have been cloned, we will need to know what sort of sampling effort of the colonies is required to fully characterize the taxonomic composition of a sample. We tested this empirically by screening a large number of colonies during initial tests and defining a minimum sample number as the point of diminishing returns where the number of unique clones detected begins to asymptote.

We amplified 16S rRNA from 10 species of cnidarians (4 Anthozoa, 3 Hydrozoa & 3 Scyphozoa) using the general cnidarian primers. These PCR products were then pooled creating a final mixture containing approximately 5-10 ng of DNA from each species. These pooled 16S rRNA fragments were then cloned using a TOPO TA cloning kit (Invitrogen Life Technologies, Carlsbad, CA) following the manufacturer's instructions. Two volumes of bacterial suspension were plated (50 and 100 µL). After incubation at 37°C overnight, positive colonies were picked for further analysis. A voucher plate of these clones was created while DNA was isolated from

the remainder of the colony. For the DNA extraction, each colony was placed into 50 µl of sterile water, boiled for 15 minutes and then stored at -20°C until use. Universal plasmid primers were used in a PCR to verify that the clones contained an insert. This amplification product (0.2 µl for selective PCR or 10 µl for RFLPs) was then used as the template in subsequent tests to identify the species represented by the cloned DNA.

Detection limits

We realize that cnidarians are relatively uncommon members of the ballast water community. Thus, we needed to ascertain that our primers would be able to detect a small number of individuals (their DNA) in a given sample. The lower detection limits of this protocol were evaluated by using a series of samples with known concentrations of target DNA. A dilution series of DNA from several species of cnidarians was prepared and then tested for amplification. Final DNA concentrations in these PCR reactions were as follows: 30 ng, 12 ng, 6 ng, 3 ng, 1 ng, 500 pg, and 250 pg. We tested two brands of *Taq* polymerase (*Taq* in Buffer B [Promega Co., Madison, WI] and *TaqPlus Maxx* [Stratagene, La Jolla, CA]) for their ability to amplify these quantities of DNA.

Testing mixed samples

In addition to determining the lower detection limits of this protocol, and we needed to be certain that the primers will be able to detect a small number of individuals (their DNA) among a much larger pool of DNA from other organisms. We selected crustaceans and annelids as the background against which to test the detection ability of the protocol. Two taxa were used in these tests: the fairy shrimp (*Artemia* sp.) and a marine polychaete (Nereidae).

The potential for the general cnidarian primers to amplify these two species was tested under a variety of PCR conditions and DNA concentrations (500 ng, 50 ng and 10 ng). Then mixed samples of cnidarians and non-cnidarians were prepared to test the ability of the protocol to detect cnidarians in a mixed pool of DNA. DNA from four cnidarian species (2 Anthozoa & 1 each of Hydrozoa and Scyphozoa) was mixed with DNA of either the polychaete or *Artemia* for final concentrations of 0.6 ng/µl and 10 ng/µl respectively.

Phase III. Evaluate markers with ballast water samples.

We tested our protocol on one set of ballast water samples provided by Dr. Eric Holm of the Naval Surface Warfare Center that were collected as part of his research on ballast water organisms. These samples were collected on February 11, 2004 from the single-hulled oil tanker the USNS Grumman (T-AO 195). The Grumman had just arrived in port from the Mediterranean and had conducted a triple empty-refill exchange in the open ocean while en route on February 7. Some large zooplankton organisms were observed in the initial samples (E. Holm, pers. comm.) so these tanks were sampled again for the purposes of this study. A 20µm plankton net was towed through one time each through tanks 2P and 2S at a rate of 0.5 m/s. The contents of the net from tank 2P were placed into about 250 ml of sea water while the contents from tank 2S were placed into 250 ml of 100% ethanol. The samples were then placed into a cooler with blue ice packs and express mailed to our laboratory where they arrived on February 13 and processed that same day.

The ballast water sample was first filtered through a 0.45 μm Nalgene MF75 sterile filtration unit (Rochester, New York). Material remaining on top of the filter membrane was collected with a pipette, and approximately 100 μl of this material was placed into a 1.5 ml tube. DNA was extracted from these samples using two previously described methods (DNAzol and Qiagen). The quality of the extracted DNA was examined by gel electrophoresis on 1% agarose gels, stained with ethidium bromide (0.5 μg/ml) and then visualized under ultraviolet light.

In addition to the ballast water samples we used environmental plankton tows as mock ballast water samples. On January 12, 2004 plankton tows were taken in Mississippi Sound just north of one of Horn Island. A 20μm plankton net was towed over a distance of approximately 5 meters a total of 20 times. The material collected in the net was placed into about 500 ml of sea water and returned to the lab in a cooler the same day. The samples was then stored at 4°C until it was processed the following day. In addition, material remaining after processing was preserved in 100% ethanol, and then used in a DNA extraction after one month of storage.

We were not certain if either the ballast water or environmental samples would actually contain cnidarians. In order to test the ability of our methods to detect single individuals of planktonic cnidarians we decided to use individual *Hydra* as surrogates in our protocols. *Hydra* is a genus of freshwater hydrozoan that is typically about 0.3-1cm in length. We obtained living individuals of *Hydra* from Carolina Biological Supply Co., and DNA extractions were performed on single individuals using the previously described methods. The quality of the DNA was assessed as previously described.

DNA from the ballast water, environmental samples and individual *Hydra* was used in PCR with the general cnidarian primers using the reaction conditions previously described and an annealing temperature was 40°C. A range of DNA concentrations was tested for each of the types of samples.

Results and Accomplishments

Phase I. Characterize the molecular markers needed for identifications.

Specimens

A total of 26 species consisting of 10 anthozoans, 9 hydrozoans, 6 scyphozoan and 1 cubozoan were acquired for this study (Table 1). The geographic location of these collections included the Gulf of Mexico and northern Atlantic.

DNA isolation

We found that fresh tissue produced the best extractions, although preserved tissues usually also worked well. All three extraction methods seemed to give similar results, so for most of the samples we used two of the simpler methods (Dneasy kit and the DNAzol method). DNA extractions consisted of high molecular weight DNA, and concentrations typically ranged from 200-400 ng/μl.

Marker Identification

We used sequences from GenBank as well as any sequences that we generated in our marker selection process. The goal again was to identify a DNA sequence or sequences that would provide the appropriate taxonomic resolution. This marker should provide a means to distinguish among the major taxonomic groups of cnidarians and also contain enough variation to distinguish among species within groups.

18S rRNA.— Numerous sequences for the 18S rRNA were available on GenBank for each of the major groups of cnidarians (Table 2). For the Hydrozoa, we examined 45 GenBank sequences and three of our own. The Anthozoa were similarly well represented with 38 GenBank sequences and five of our own. For the Scyphozoa there were only 14 GenBank sequences and we added four of our own, and the Cubozoa also had limited data available with nine GenBank sequences and one of our own.

After analysis of the sequence alignments both within and between taxonomic groups, we rejected 18S rRNA as a marker for use in this study. This gene lack the right mix of conserved and variable regions that would enable us to design robust taxa-specific primers. While there were conserved regions within groups, they were also tended to be conserved across the groups. Furthermore, the variable regions between groups also tended not to be conserved enough within groups.

16S rRNA.— GenBank provided many sequences of 16S rRNA for each of the major groups of cnidarians (Table 3), although not as many as found for 18S rRNA. The Anthozoa were the best represented on GenBank of which we used 75 sequences and added eight of our own. For the Hydrozoa, we examined five GenBank sequences and six of our own. We found six sequences on GenBank for the Scyphozoa and used six of our own. There was only one sequence on GenBank for the Cubozoa.

After comparing the 16S rRNA sequences within and among the various taxonomic groups, it was deemed to be useful as a marker for our protocol. We were able to identify conserved regions that differed among in the group, which provided us a location to the design

diagnostic primers. Also, the variability within each group seemed sufficient distinguish among species or at least genera.

ITS.— The few sequences that we obtained from GenBank or generated from our samples demonstrated a great deal of variation (size and sequence) among and within groups. After the identification of the 16S rRNA gene as an appropriate marker, we did not pursue any additional work on ITS. However, the high degree of within group variation, would seem to indicate that it would make a useful secondary marker in obtaining accurate species identifications.

Taxa-specific primers

We designed eight primers for 16S rRNA that were intended to specifically amplify major taxonomic groupings of cnidarians (Table 4; Figure 1). The goal was to be able to distinguish among the three major groups of anthozoans (Alyconaria - soft corals; Zoantharia-Actiniaria - anemones; Zoantharia-Scleractinia - hard corals), hydrozoans and scyphozoans. Cubozoans are not particularly common in temperate marine waters and there is not much data available so we did not include them in our protocol development. We tested the selective amplification ability of these primers in the following fashion. General cnidarian 16S rRNA primers (previously described) were used to amplify representative individuals from the major groups. This material (0.2 μl PCR product) was then used as the template in PCR using the taxa-specific primers. PCR conditions are the same as described above with an annealing temperature of 60°C.

Unfortunately, the taxa-specific primers were not all equally effective. The hydrozoa and scyphozoa primers tended to amplify anthozoan groups. To get around this problem in our protocol, it was necessary to sequentially test samples - first with Anthozoa primers, and then any samples not identified as such were tested with the Hydrozoa and Scyphozoa primers. The testing scheme was as follows:

1. AlcyonariaF & ActiniariaF & ActiniariaR. This produced two bands for Alcyonaria samples and 1 band for Actiniaria.
2. ScleractiniaF & ActiniariaR. This primer combination only amplified the Scleractinia.
3. Remaining samples were identified as either Hydrozoa or Scyphozoa by testing them in two reactions with the HydrozoaF & HydrozoaR and ScyphozoaF & ScyphozaR primer pairs.

An example of this sequential amplification approach is presented in Figures 2 and 3. First a group of unknown clones is tested with the AlcyonariaF & ActiniariaF & ActiniariaR primers (Fig. 2). In this case, one clone produced two bands (i.e. Alcyonaria anthozoan) and nine clones only produced one band (Actiniaria anthozoan). The remaining four clones did not amplify indicating that they were either Hydrozoa or Scyphozoa. Clones that were identified as either Hydrozoa or Scyphozoa were next subjected to PCR with both the Hydrozoa F-R and Scyphozoa F-R primer sets. In this example (Fig. 3), thirteen clones were amplified with both sets of primers. Five clones amplified using the hydrozoan but not the scyphozoan primers, while the remaining eight amplified with the scyphozoan but not the hydrozoan primers.

RFLPs of 16S rRNA

The goal of the RFLP approach was to identify taxa within each major taxonomic grouping to the level of genus or species. Depending on the group, these diagnoses required the

use of either a single enzyme to several enzymes in combination. For example, for nine Scyphozoa, a single enzyme (*Alu*I - Table 5) produces five unique haplotypes. In cases like this, the method of resolving these RFLP haplotypes is straightforward and usually easy to interpret. For example, one of two restriction enzymes (*Ase*I & *Dpn*II) easily distinguishes between the hydrozoan taxa *Tubularia* and *Campanularia* (Fig. 4). The location of these particular restriction enzymes in these two species produces a characteristic banding pattern.

However in some cases, such as *Phyllorhiza punctata* and *Rhopilema verilla*, two species may have the same haplotype for this enzyme, but can be distinguished by using a second enzyme (*Ase*I). But in some cases, two taxa may be identical for all of the restriction enzymes being considered. Among eight Hydrozoa (Table 6), *Ase*I produces 7 unique haplotypes. *Bougainvillia carolienensis* and *Liriope tertraphylla* are identical at *Ase*I as well as the four other enzymes presented in Table 6.

The lack of complete taxonomic resolution was more prevalent among the Anthozoan taxa. We examined the RFLP patterns for a subset of Anthozoa including the Alyconaria group with the order Alcyonacea (soft corals) and Gorgonacea (sea pens) and the order Actiniaria (sea anemones). Our sample of Alcyonacea included 18 species representing 11 families. Using three enzymes resulted in nine distinct composite haplotypes (Table 7) where seven were unique to a single species and two were found in multiple species. Similarly in the 19 species of Actiniaria, representing five genera, we were able to identify 11 unique composite haplotypes using three enzymes (Table 8). This lack of taxonomic resolution is likely the consequence of several things. Both groups of Anthozoa include multiple representatives of the same genus. Given that mitochondrial DNA in Anthozoa has been shown to evolve at a slower rate than other organisms, it is not surprising that congeners would not always be divergent enough to possess unique haplotypes. Admittedly, the inclusion of additional restriction enzymes in our RFLP survey could possibly identify unique cutting enzymes, but we were hopping to limit this to a manageable number. While not all species are identifiable, a substantial proportion are (e.g. 50% and 58% in our example). Should a more refined estimate of the numbers of species be required other techniques such as single-strand conformation polymorphisms (SSCPs) and denaturing gradient gel electrophoresis (DGGE) might be employed. However, these approaches are more technically challenging, time consuming and expensive.

Phase II - Lab evaluation of markers - identifications & quantification

Detection ability

Cloning the 16S rRNA sample mix of ten cnidarian species, produced an ample number of positive colonies. We randomly selected 55 colonies and attempted to identify the taxa represented using selective primers followed by RFLP analysis. In order to assess the relative sampling effort required to characterize the taxonomic composition of the sample, we plotted the number of colonies examined versus the number of species detected (Fig. 5). The total number of species recovered increased relatively rapidly as more clones were sampled, however by the end of the sampling one species (a hydrozoan) had yet to be detected. When the taxonomic composition of the clones was examined, anthozoans were the most readily recovered group making up the bulk of the clones (Fig. 6). However, even though relatively few hydrozoan or

scyphozoan clones were detected, their numbers had almost reached a maximum after about half way into the sampling.

The fact that mostly anthozoan species were detected in the mixed pool could be a function of two things. First, the starting concentrations of DNA likely were greater for the anthozoan species used in our test. That is not a problem for the protocol, and in fact provides a means of roughly quantifying the number of a given species in the initial sample. A second explanation might be that the general cnidarian primers may have selectively amplified the anthozoan DNA, which led them to be over represented among the PCR products that were cloned. This idea needs further testing before we can safely use the number of clones recovered as an estimate of the number of individuals (or concentration of their DNA) in the original sample.

Detection limits

We compared the ability of two brands of *Taq* polymerase to amplify DNA of *Metridium senile*, *Epicystis crucifer*, *Cassiopea xamachama* and *Cyanea capillata* at a variety of combinations. Not all concentrations were tested with both brands. PCR conditions were the same as previously described except that annealing temperatures were either 40° or 55°C. Promega brand *Taq* seems robust in amplifying concentrations down to the level of 3 ng, but provides weaker amplification down to the level of 500 pg. In contrast, *TaqPlus Maxx* provided the greatest sensitivity as strong amplifications were obtained down to the level of 250 pg of DNA (Table 9; Figure 7).

Testing mixed samples

The general cnidarian 16S rRNA primers were tested for their ability to amplify non-cnidarian DNA at three concentrations (500 ng, 50 ng and 10 ng) using the Promega *Taq* and the *TaqPlus Maxx*. At an annealing temperature of 55°C using the *TaqPlus Maxx*, there was no amplification of *Artemia* or the polychaete. However, with the Promega Taq there was faint amplification of the polychaete at the two higher DNA concentrations.

Our mixed cnidarian/non-cnidarian PCR reactions consisted of 0.6 ng each of *Metridium senile*, *Epicystis crucifer*, *Cyanea capillata* and *Tubularia* and 10 ng of either *Artemia* or polychaete. We found that the general cnidarian primers were able to amplify the cnidarian template DNA in these mixed samples when using the *TaqPlus Maxx* and an annealing temperature of 55°C. A range of sizes of PCR product was detected, suggesting that the PCR was successful at amplifying more than one species of cnidarian from the mix.

Phase III - Testing the protocol on a ballast water sample

DNA extractions

Upon arrival at the lab, we inspected the ballast tank samples from the USNS Grumman under a dissecting microscope. Very few individuals of zooplankton were evident in either the fresh or ethanol preserved samples. After filtration of the samples, we ran three duplicate extractions of the fresh and ethanol preserved samples using each of the extraction protocols. No DNA was detected by the agarose gel check of these extractions (Figure 8). In the environmental plankton tows there was abundant life, although most of it appeared to be phytoplankton. We ran four duplicate extractions of each protocol for the fresh material and one extraction of each protocol using the ethanol preserved material. DNA was detected for each of these extractions (Figures 8 & 9). Both procedures yielded high molecular weight, although the

DNAzol method appeared to provide slightly better quality DNA. The DNA extractions of individual *Hydra* were successful using both methods, but the best yields seemed to be obtained with the Qiagen method (Figure 10).

The comparison of the DNA extractions from the ballast tank, environmental plankton tow and individual *Hydra* suggest that the lack of DNA in the ballast tank samples was the result of the sparse number of organisms in the sample. The fact that extractions of individual *Hydra* yielded detectable amounts of DNA indicates that our methods can obtain DNA from small individuals and/or small numbers of individuals.

Whether or not the sample is fresh or preserved does seem to have an impact on the quality of the DNA obtained. However, this observation is tentative and is subject to additional testing. If it holds, then the recommendation for a sampling protocol would be to use fresh samples whenever possible.

PCR results

Attempts to amplify the DNA extractions from the ballast water samples were not successful at any of the DNA concentrations used. This was not too surprising as there was not detectable DNA in any of the extractions. However, one of the strengths of the PCR method is that it can amplify trace amounts of target DNA under the right conditions. One way to explain the absence of amplifications in the ballast water samples is that there were no cnidarians present. The environmental plankton tow samples also did not produce any amplifications. However, the individual *Hydra* produced robust amplifications. Since we were able to amplify individual cnidarians (*Hydra*), this might suggest that cnidarians were also absent in the environmental samples.

One additional explanation for the lack of amplification in either the ballast water or environmental samples is that PCR inhibitors were present or that nontarget DNA may have prevented amplification of target DNA. This did not seem to be a problem with the trials using *Artermia* and polychaete DNA mixed with cnidarian DNA, but to exclude this possibility we performed one additional test. We mixed DNA from one Hydra extraction with equal volumes from the ballast water and environmental samples. This was then used as a template for an amplification using the standard conditions. In both cases successful amplifications were obtained indicating that inhibitors or nontarget DNA was not the cause of the failed PCR.

Conclusions

Summary, Utility and Follow-on Efforts

In recent years, molecular techniques have been increasingly used to address applied ecological problems. With regard to the question at the heart of this project, the identification and quantification of ballast water organisms, molecular techniques provide the only the realistic alternative to traditional approaches. The difficulty in finding individuals with the considerable technical expertise required to classify planktonic organisms is compounded by the simple fact that many larval organisms are morphologically indistinguishable even to the best trained eye. Of course, we do not intend to suggest that a molecular approach is an easy answer to these problems. While the technical skills are easy to acquire, there is considerable difficulty in designing a robust and generalized molecular protocol to effectively monitor ballast water transport of exotic species. This challenge is made clear by the current lack of the application of these approaches to this very important issue.

This project has been successful in taking the first step in bridging the gap between the potential and the application of molecular techniques. Within the constraints of the lab we have been able to demonstrate the practicality and utility of our protocol to detect, identify and quantify a specific group of organisms (cnidarians) within a ballast water sample. Specifically we were able to accomplish the following:

Marker Identification

- Identified the mitochondrial 16S rRNA gene as an appropriate molecular marker,
- Design primers to selectively amplify major groups of cnidarians,
- Identify RFLPs that were mostly diagnostic with the exception of some anthozoan species,

Detection Ability

- Substantiate the usefulness of the PCR and clone approach in detecting species in a mixed sample,
- Determined the detection limits of PCR minimally extends down to the level of 250 pg of DNA, and
- Demonstrated the ability of the PCR to detect target DNA in a mixture with nontarget DNA.

While the developmental process and lab tests were quite promising, as of yet we have been less successful in carrying these techniques from the lab over to actual field samples. However, we feel that it would be premature to give up on the protocol just yet. A brief summary of our results and rationale for our expectations follows. For the set of ballast tank samples that we received, we were unable to detect any DNA in our extractions. As previously mentioned, this is likely a consequence of the very low density of organisms present in the samples. We find this explanation reasonable, as in our plankton tow samples we were able to extract copious amounts of DNA. However, despite the high quality DNA obtained from the plankton tow samples we were unable to amplify any cnidarians from these samples. Again, the explanation could simply be that there was few or no cnidarians present. But we are unable to confirm this as we did not microscopically examine the species present in these samples. However, we were quite successful in our work with surrogates of small planktonic cnidarians (the *Hydra*). Amplifications of extractions of single *Hydra* as well as mixtures of *Hydra* and plankton sample DNA were all successful. This suggests to us that had cnidarians been present in the plankton tow samples, then they would have been detectable by our protocol.

There are several follow on efforts that could been undertaken to better develop the protocol presented in this report. First and foremost would be to obtain additional ballast tank samples, with a larger number of taxa present. We have been in contact with our source of samples (Dr. Eric Holm) and he is willing to provide us with additional samples as he continues with his project. When we acquire these new samples, we will test them as is and in addition spike a few of the extractions with various numbers of *Hydra*. These spiked samples will serve as positive controls, representing samples known to have some cnidarians present. As the field validation of our protocol is the only accomplishment that we lack, we are quite anxious to work up additional samples. Based the robustness of our lab tests we remain confident that our protocol will be successful for its intended purpose providing we test it under the appropriate conditions.

The second follow up effort would be to refine our ability to distinguish among the taxa using our marker. While the RFLPs are robust for the most part, they were not quite as diagnostic of the Anthozoa as we had hoped. We had initially focused on a RFLP approach because it is technically simple and relatively inexpensive. However, we recognize that is does not have the discriminatory ability that other techniques posses. For example, both SSCPs and DGGE are theoretically able to detect one or two base pair differences between two sequences. If the additional tests of field samples described above proved promising, then it would be useful to explore these methods and potentially add the to our protocol.

Economic Feasibility

As far as the economic feasibility of our protocol, below we provide an estimate of the cost to process a ballast water sample from a single vessel. These cost estimates with regard to reagents and time are fairly robust, but the total cost would depend on how many samples would need to be processed from a single vessel. Also, unfortunately, we are unable to provide a comparison with the costs currently associated with identifying species in a ballast water sample by traditional means.

The overall cost in reagents should be roughly under $200, with the step by step cost estimates as follows:

1. Ballast water sample DNA extraction - 1-2 filtrates - $20
2. PCR with general cnidarian primers - $1
3. Cloning reactions (2) - $60
4. Clone verification (50-100 clones) - $10-$20
5. Taxa identification PCR - $50-$100
6. RFLPs - $15-$30

The time required to process a sample from start to finish would be about eight days for a single technician. However, each task would not necessarily consume the technician's time for the entire day so multiple samples could be in the work flow simultaneously.

Day 1 - Extract DNA from ballast water samples / initial PCR.
Day 2 - Cloning.
Day 3 -Clone DNA extraction / PCR verify positive clones.
Day 4 - PCR verification of positive clones continued.
Day 5 -Taxa identification PCR.

Day 6 - Taxa identification PCR continued.
Day 7 - RFLP identifications.
Day 8 - RFLP identifications continued.

An assessment of the attractiveness of this technology must be based on the need/desire for a certain level of taxonomic resolution in identifications. At the grossest taxonomic level, visual identification are certainly the best and easiest. For example, if you simply wanted to know the relative numbers of crustaceans vs. mollusks. But beyond the level of family in many cases, the molecular approach would probably win out due to the high taxonomic skill level required by the technician as well as the robustness of taxonomic identifications a molecular approach would provide.

One additional application of this protocol that will warrant examination is its use in identifying hull fouling organisms. Once again taxonomic designations for these organisms may be difficult, especially if the specimens are damaged in the act of removing them. However, as long as they are preserved appropriately, this protocol can be used to identify them based on their DNA.

Transition Plan

The information about the protocol will be conveyed to the scientific community in a peer-reviewed publication. By getting the concept and methodology of our protocol out into the literature we hope that we can stimulate interest and promote evaluation by other researchers who will be able to test and validate the feasibility and rigor of the methodology for themselves. This process will certainly lead to improvements in the protocol when it is applied to novel situations beyond the ones we devised for testing in the developmental stages.

While the cost assessment that we have provided is rather rough, we will point out that the deliverable aspect of this project (a means to identify cnidarians in ballast water) is a product that is not realistically obtained by any other method. Another deliverable aspect of this project will be the demonstration of a protocol that, given suitable development, can be applied towards the identification of any of the groups of organisms commonly found in ballast water or in a hull fouling community.

Recommendations

Since this project was designed to demonstrate a proof of concept, we are not necessarily at the stage where our deliverable product can be immediately transitioned into an operational format. However, with some additional testing of actual ballast samples under a variety of conditions we will be in a position to better evaluate the future of our protocol. Similarly, the basic methodology, once in the literature, may subsequently be developed by others for other taxonomic groups that may be of special interest or relevance.

Literature Cited

Bernhard, A.E. and K.G. Field. 2000. Identification of non point sources of fecal pollution in coastal waters using host-specific 16S ribosomal DNA genetic markers from fecal anaerobes. Applied and Environmental Microbiology 66(4):1587-1594.

Bernston, E.A., S.C. France, and L.S. Mullineaux. 1999. Phylogenetic relationships within the Class Anthozoa (Phylum Cnidaria) based on nuclear 18S rDNA sequences. Molecular Phylogenetics and Evolution 13:417-433.

Bernston, E.A., F.M. Bayer, A.G. McArthur and S.C. France. 2001. Phylogenetic relationships within the Octocorallia (Cnidaria: Anthozoa) based on nuclear 18S rRNA sequences. Marine Biology 138:235-246.

Bilodeau, A.L., W.S. Lankford, T.J. Kim, D.L. Felder and J.E. Neigel. 1999. An ultrasensitive method for detection of single crab larvae (*Sesarma reticulatum*) by PCR amplification of a highly repetitive DNA sequence. Molecular Ecology 8:681-684.

Borneman, J. and R.J. Hartin. 2000. PCR primers that amplify fungal rRNA genes from environmental samples. Applied and Environmental Microbiology 66(10):4356-4360.

Carlton, J.T. 1996. Pattern, process, and prediction in marine invasion ecology. Biological Conservation 78:97-106.

Carlton, J.T. and J.B. Geller. 1993. Ecological roulette: the global transport of nonindigenous marine organisms. Science 261:78-82.

Chomczynski P., K. Mackey, R. Drews and W. Wilfinger. 1997. DNAzol: A reagent for the rapid isolation of genomic DNA. BioTechniques 22:550-553.

Collins, A.G. 2002. Phylogeny of Medusozoa and the evolution of cnidarian life cycles. Journal of Evolutionary Biology 15:418-432.

Cunningham, C.W. and L.W. Buss. 1993. Molecular evidence for multiple episodes of paedomorphosis in the family Hydractiniidae. Biochemical Systematics and Ecology 21:57-69.

Deagle, B.E., N. Bax, C.L. Hewitt and J.G. Patil. 2003. Development and evaluation of PCR-based test for detection of *Asterias* (Echinodermata: Asteroidea) larvae in Australian plankton samples from ballast water. Marine and Freshwater Research 54:709-719.

Diekmann, O.E., R.P.M. Bak, W.T. Stam and J.L. Olsen. 2001. Molecular genetic evidence for probable reticulate speciation in the genus *Madracis* from a Caribbean fringing reef slope. Marine Biology 139:221-233.

France, S.C., P.E. Rosel, J. Ewann Agenbroad, L.S. Mullineaux and T.D. Kocher. 1996. DNA sequence variation of mitochondrial large-subunit rRNA provides support for a two-subclass organization of the Anthozoa (Cnidaria). Molecular Marine Biology and Biotechnology 5:15-28.

Geller, J.B. and E.D. Walton. 2001. Breaking up and getting together: evolution of symbiosis and cloning by fission in sea anemones (Genus Anthopleura). Evolution 55:1781-1794.

Grosholz, E.D. and G.M. Ruiz. 1995. The spread and potential impact of the recently introduced European green crab, *Carcinus maenas*, in central California. Marine Biology 122:239-247.

Hillis, D.M. and M.T. Dixon. 1991. Ribosomal DNA: molecular evolution and phylogenetic inference. Quarterly Review of Biology 66:411-453.

Larsen, K.M., H.M. Perry, C. Trigg and T. VanDevender. 2001. Fishery implications of *Phyllorhiza punctata* blooms in Mississippi coastal waters. Journal of the Mississippi Academy of Sciences 46:63.

MacIsaac, H.J. 1996. Potential abiotic and biotic impacts of zebra mussels on the inland waters of North America. American Zoologist 36:287-299.

MaKinster, J.G., J.E. Roberts, D.L. Felder, C.A. Chlan, M. Boudreaux, A.L. Bilodeau and J.E. Neigel. 1999. PCR amplification of a middle repetitive element detects larval stone crabs (Crustacea: Decapoda: Menippidae) in estuarine plankton samples. Marine Ecology Progress Series 188:161-168.

McFadden, C.S., I. Tullis, M.B. Hutchinson and K. Winner. 2000. Rates of evolution of cnidarian mitochondrial genes. American Zoologist 40:1124

Medlin, L., H.J. Elwood, S. Stickel and M.L. Sogin. 1988. The characterization of enzymatically amplified eukaryotic 16S-like rRNA-coding regions. Gene 71491-499.

Michotey, V., V. Méjean and P. Bonin. 2000. Comparison of methods for quantification of cytochrome cd_1-denitrifying bacteria in environmental marine samples. Applied and Environmental Microbiology 66(4):1564-1571.

Pace, N.R. 1997. A molecular view of microbial diversity and the biosphere. Science 276:734-740.

Palumbi, S., B. Kessing and A. Martin. 1991. The Simple Fool's Guide to PCR, Version 2.0 Zoology Department, Honolulu, Hawaii.

Rodriguez-Lanetty, M. and O. Hoegh-Guldberg. 2002. The phylogeography and connectivity of the latitudinally widespread scleractinian coral *Plesiastrea versipora* in the western Pacific. Molecular Ecology 11:1177-1189.

Romano, S.L. and S.R. Palumbi. 1996. Evolution of scleractinian corals inferred from molecular systematics. Science 271:640-642.

Ruiz, G.M., J.T. Carlton, E.D. Grosholz and A.H. Hines. 1997. Global invasions of marine and estuarine habitats by non-indigenous species: mechanisms, extent and consequences. American Zoologist 37:621-632.

Schroth, W., G. Jarms, B. Streit and B. Schierwater. 2002. Speciation and phylogeography in the cosmopolitan marine moon jelly, *Aurelia* sp. BMC Evolutionary Biology 2:1.

Seutin, G., B.N. White, and P.T. Boag. 1991. Preservation of avian blood and tissue samples for DNA analyses. Canadian Journal of Zoology 69:82-90.

Shearer, T.L., M.J.H. Van Oppen, S.L. Romano and G. Worheide. Slow mitochondrial DNA sequence evolution in the Anthozoa (Cnidaria). Molecular Ecology 11:2475-2487.

Smith, L.D., M.J. Wonham, L.D. McCann, G.M. Ruiz, A.H. Hines and J.T. Carlton. 1999. Invasion pressure to a ballast-flooded estuary and an assessment of inoculant survival. Biological Invasions 1:67-87.

Weekers, P.H.H., R.J. Gast, P.A. Fuerst and T.J. Byers. 1994. Sequence variations in small-subunit ribosomal RNAs of Hartmannella vermiformis and their phylogenetic implications. Molecular Biology and Evolution 11:684-690.

White, T.J., T. Bruns, S. Lee and J. Taylor. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, pp. 315-322. *In* M.A. Innis, D.H. Gelfand, J.J. Sninksy and T.J. White (eds.), *PCR Protocols*. Academic Press, New York.

Appendix A - Supporting Data

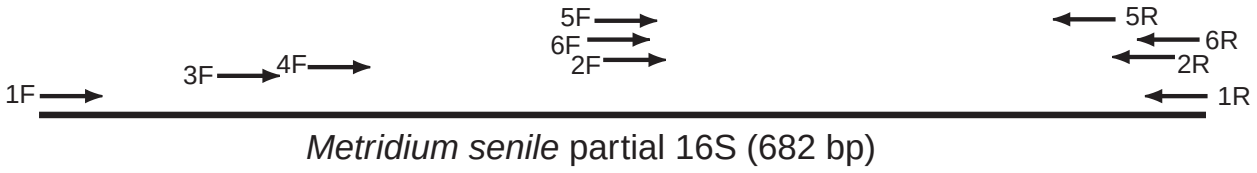

Figure 1. Positions of the taxa-specific primer pairs relative to the 16S rRNA sequence from the anthozoan *Metridium senile*. Primers are identified as follows:
1F = 16Sar; 1R = 16S-H5'; 2F & R = ActiniariaF & R; 3F = AlcyonariaF; 4F = ScleractiniaF; 5F & R = HydrozoaF & R; 6F & R = ScyphozoaF & R.

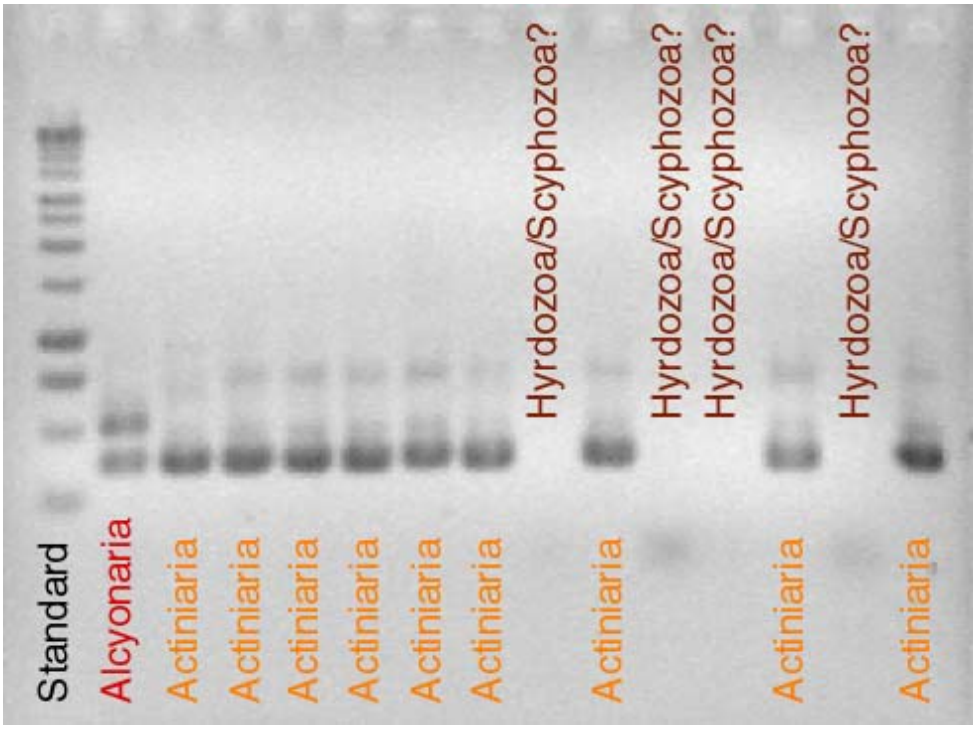

Figure 2. Amplification of 14 clones with the Alcyonaria F, Actiniaria F and R primers. The leftmost lane is a 1kb size standard (Promega). The presence of two amplification products is diagnostic of an Alcyonaria anthozoan while one major amplification product represents an Actiniaria anthozoan. Failure to amplify using these primers is taken to mean that the clone represents either a Hydrozoa or Scyphozoa. Each lane represents a load of 5µl of PCR product.

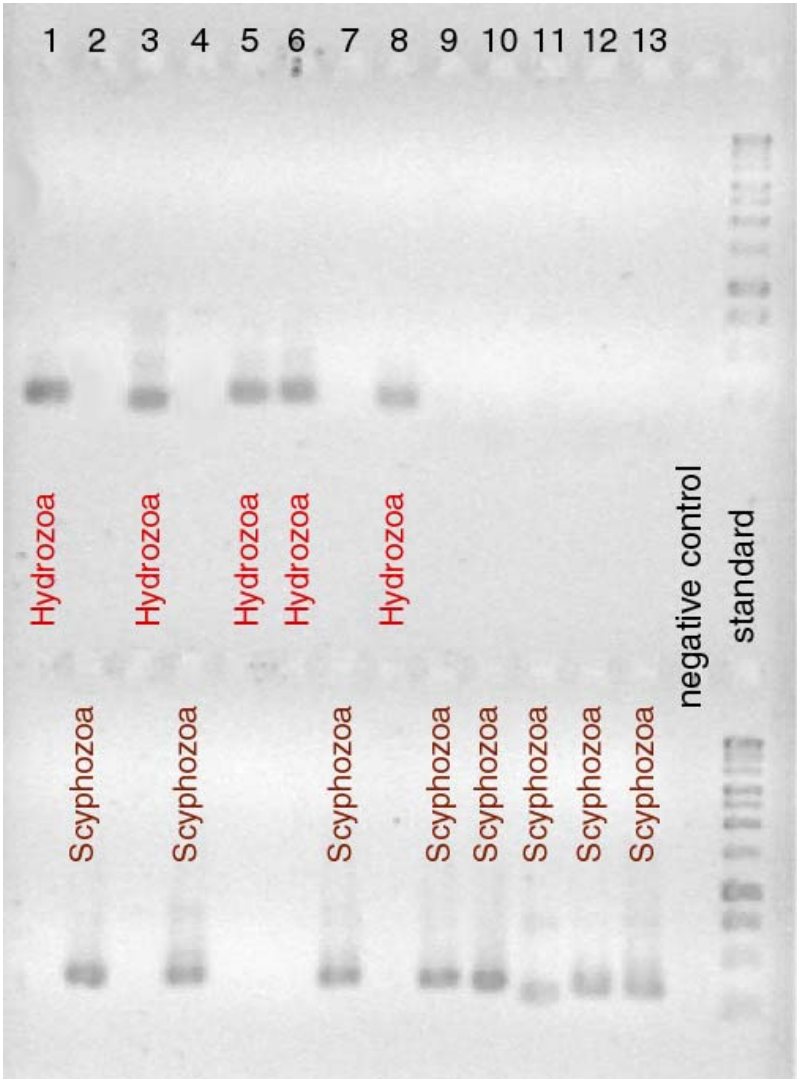

Figure 3. Two sets of amplifications of 13 clones with either the Hydrozoa F and R or Scyphozoa F and R primers. The rightmost lanes are a PCR negative control and a 1kb size standard (Promega). The presence of an amplification product with one but not the other primer set provides an identification of the clone as either Hydrozoa or Scyphozoa. Each lane represents a load of 5µl of PCR product.

Figure 4. The results of a restriction digest of two individuals each of two hydrozoan species (*Tubularia* and *Campanularia*) using *Ase*I and *Dpn*II. The different banding patterns produced by the digest (RFLP) provides a means to distinguish the two species. The rightmost lane is a 1kb size standard (Promega). Each lane represents a load of 5µl.

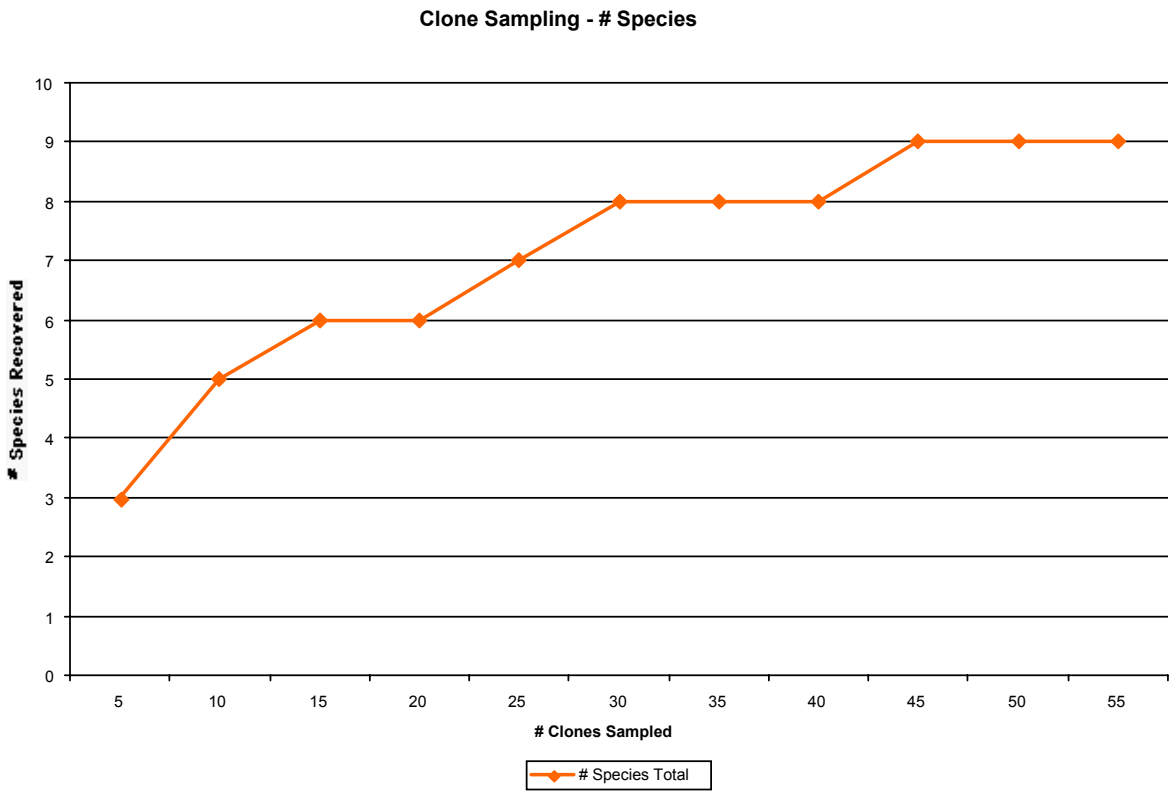

Figure 5. Analysis of the sampling effort of clones required to detect all of the species in a starting pool of mixed DNA. Plotted are the number of clones sampled versus the number of species recovered out of the initial ten.

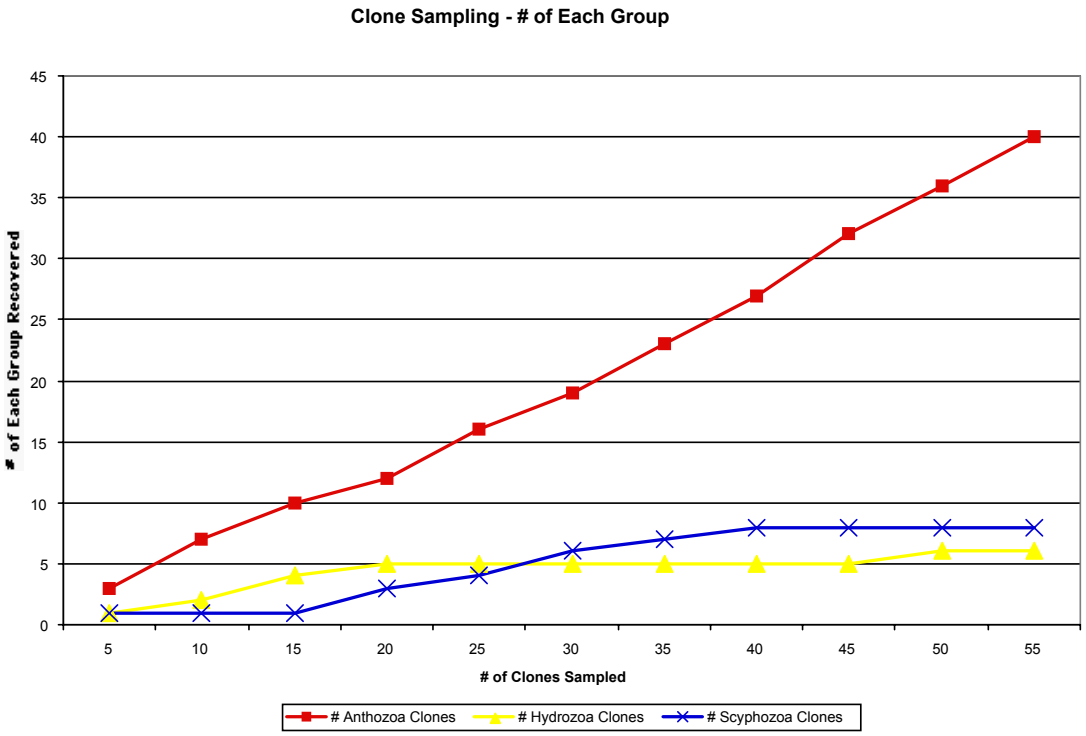

Figure 6. The breakdown of the number of clones by taxonomic group detected with increasing sampling effort.

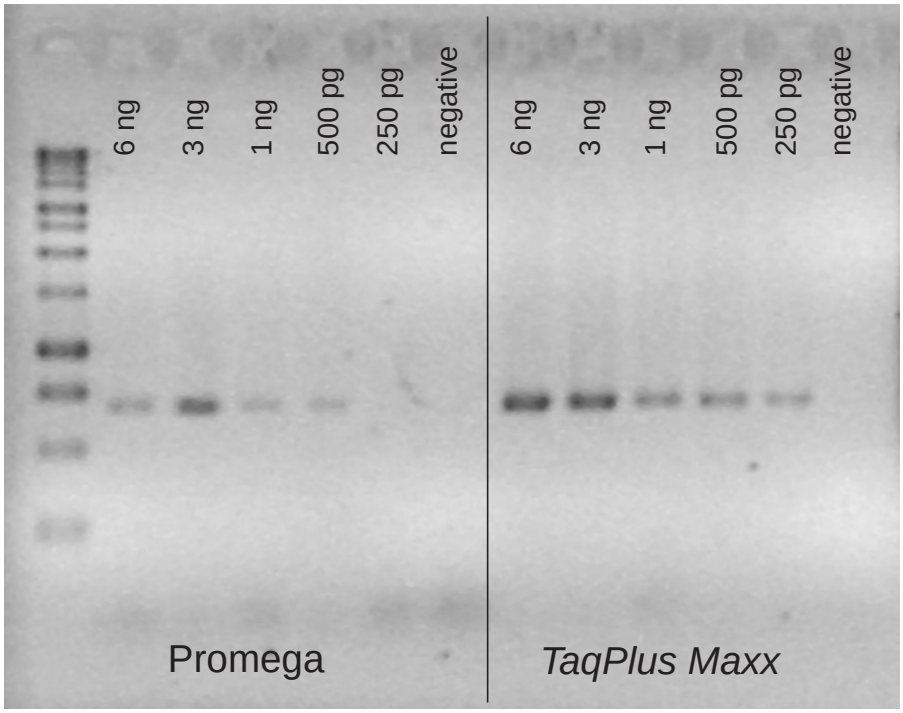

Figure 7. Comparison of the ability of two brands of *Taq* (Promega and Stratagene) to amplify DNA of *Metridium senile* at concentrations in a range of 6 ng to 250 pg. Negative signifies the negative control run with each set of reactions. The leftmost lane is a 1kb size standard (Promega). Each lane represents a load of 5µl of PCR product.

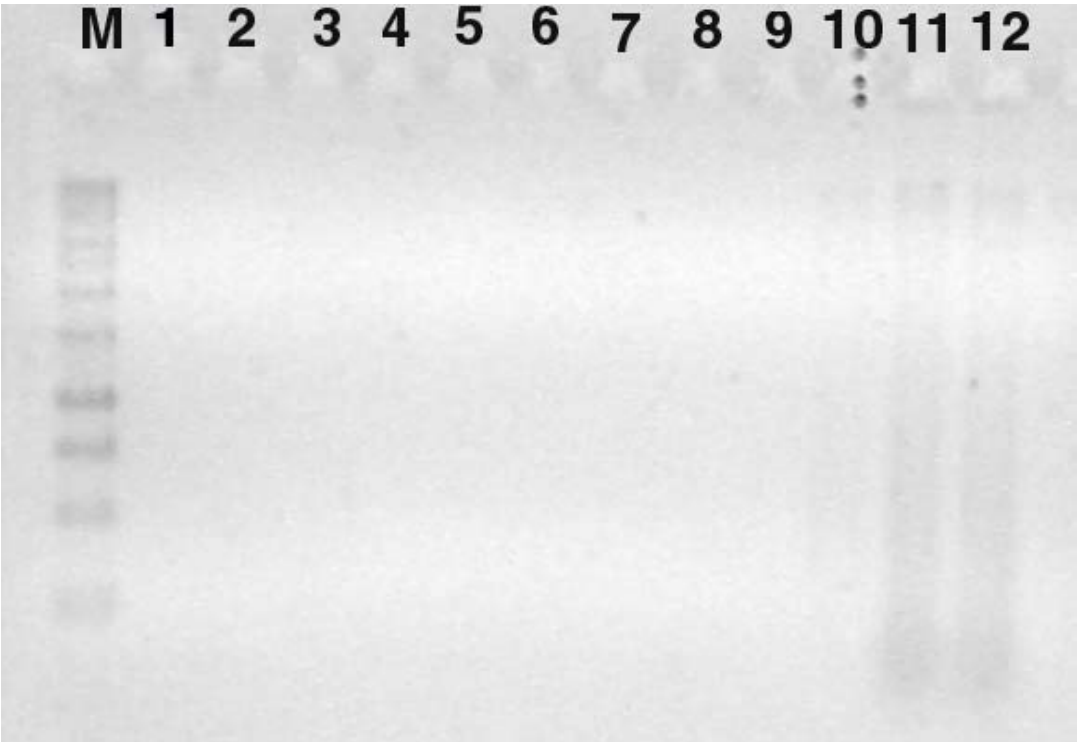

Figure 8. A gel check of the quality of DNA extractions. M identifies the size standard (Promega 1kb ladder). Lanes 1-9 are extractions from ballast tank samples (both fresh and ethanol preserved) using two different extraction techniques (Qiagen and DNAzol). Lanes 10-12 are DNA extractions from ethanol preserved plankton tow samples. Each lane represents a load of 5µl of DNA.

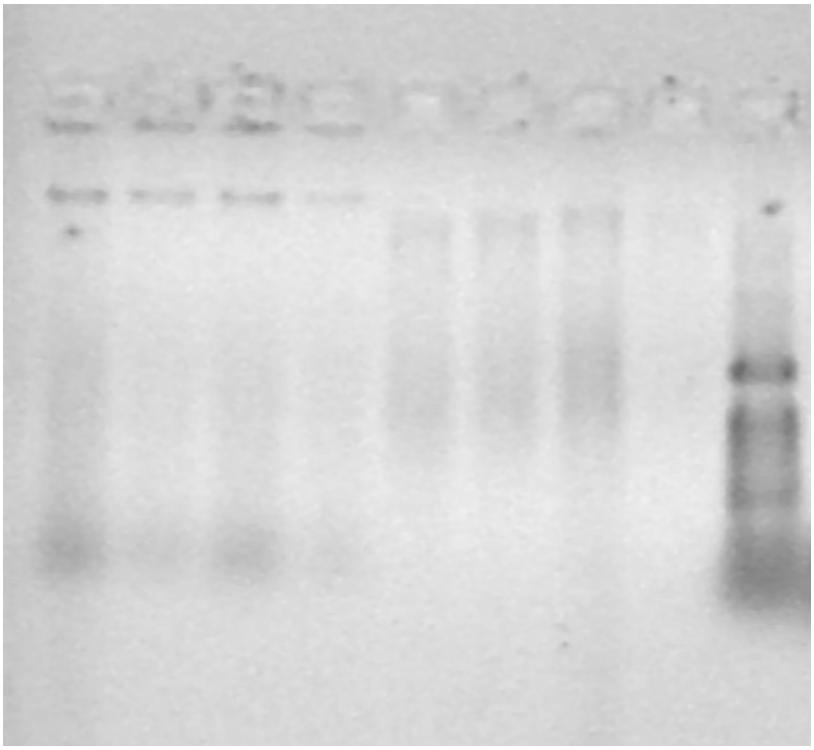

Figure 9. A gel check of the quality of DNA extractions of fresh plankton tow samples. The first four lanes are extractions with the Qiagen kit and the next four are with the DNAzol method. The last lane is a size standard (Promega 1kb). Each lane represents a load of 5µl of DNA.

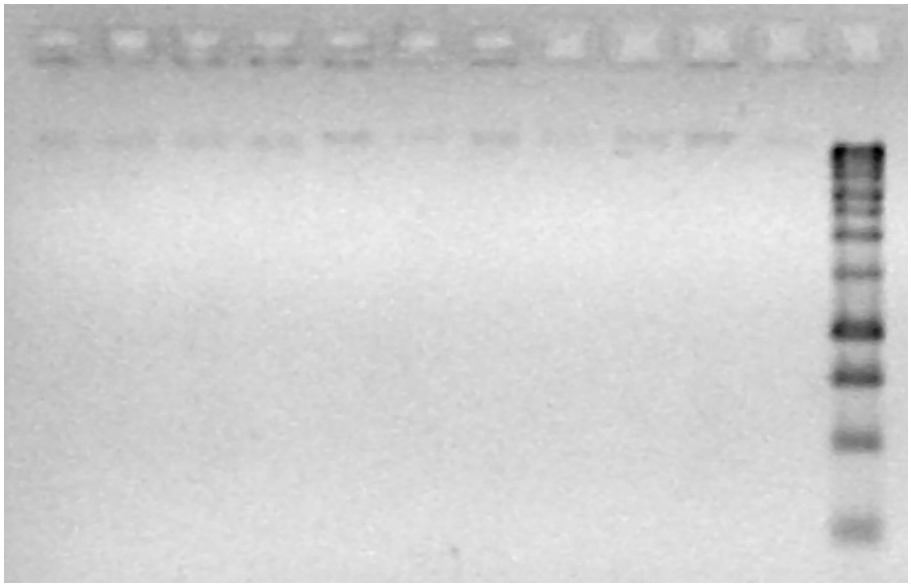

Figure 10. Gel check of individual *Hydra* DNA extractions. The last lane is a size standard. Each lane represents a load of 5µl of DNA.

Table 1. Taxonomic listing of species acquired for this study. The collection location and source is also listed. Sources are either individuals identified by name or from commercial dealers abbreviated as CBS (Carolina Biological Supply) or MBL (Marine Biological Laboratory).

Taxa	Collection locale	Source
Scyphozoa		
Rhizostomeae		
Cassiopeidae		
Cassiopea xamachama	Mississippi Sound / Florida Keys	B. Ortman / CBS
Megistiidae		
Phyllorhiza punctata	Mississippi Sound	B. Ortman
Rhizostomatidae		
Rhopilema verilla	Mississippi Sound	B. Ortman
Semaeostomeae		
Cyanidae		
Cyanea capillata	North Atlantic	MBL
Pelagiidae		
Chrysaora quinquecirrha	Mississippi Sound	B. Ortman
Ulmaridae		
Aurelia aurita	Mississippi Sound	M. Dugo/ B. Ortman
Cubozoa		
Chirodropidae		
Chiropsalmus quadromanus	Florida Keys	M. & C. Peterson
Hydrozoa		
Hydroida		
Anthomedusae		
Bougainvilliidae		
Bougainvillia carolienensis	Mississippi Sound	B. Ortman
Nemopsis bachii	Mississippi Sound	B. Ortman
Clavidae		
Clava sp.	North Atlantic	MBL
Hydractinidae		
Hydractinia echinata	North Atlantic	MBL
Hydridae		
Hydra sp.	North Carolina	CBS
Tubulariidae		
Tubularia sp.	North Atlantic	MBL
Leptomedusae		
Campanulariidae		
Campanularia sp.	North Atlantic	MBL
Sertularidae		
Sertularia sp.	North Atlantic	MBL
Siphonophora		
Cystonectae		
Physaliidae		
Physalia sp.	Gulf of Mexico	R. Darden
Anthozoa		
Alcyonaria		
Gorgonacea		
Holaxonia		

Plexauridae		
Eunicea sp.	Florida Keys	CBS
Zoantharia		
Actiniaria		
Aiptasiidae		
Bartholomea annulata	Florida Keys	CBS
Nynantheae		
Actiniidae		
Actinia equina	North Atlantic	B. Kreiser
Condylactis gigantea	Florida Keys	CBS
Hormathiidae		
Calliactis tricolor	Mississippi Sound	B. Ortman
Metridiidae		
Metridium senile	North Atlantic	MBL
Phymanthidae		
Epicystis crucifer	Florida Keys	CBS
Scleractinia		
Faviina		
Rhizangiidae		
Astrangia danae	North Atlantic	MBL
Zoanthidae		
Parazoanthidae		
Parazoanthus sp.	Florida Keys	CBS
Sphenopidae		
Palythoa caribaeorum	Florida Keys	CBS

Table 2. 18S rRNA gene sequences examined. Species are grouped taxonomically. Sequences were either obtained from GenBank (accession numbers provided) or were generated by this project (labeled as This project).

Taxa	
Scyphozoa	
Coronatae	
Atolla vanhoeffeni	AF100942
Nausithoidae	
Nausithoe rubra	AF358095
Rhizostomeae	
Cassiopeidae	
Cassiopea sp.	AF099675
Cassiopea xamachama	This project
Catostylidae	
Catostylus sp.	AF358100
Megistiidae	
Phyllorhiza punctata	This project
Rhizostomatidae	
Rhopilema verilla	This project
Stomolophus meagris	AF358101
Semaeostomeae	
Cyanidae	
Cyanea sp.	AF358097
Pelagiidae	
Chrysaora colorata	AF358098
Chrysaora melanaster	AF358099
Ulmaridae	
Aurelia aurita	This project
Aurelia aurita	U19541
Aurelia aurita	AY039208
Phacellophora camtschatica	AF358096
Stauromedsae	
Depastridae	
Craterolophus convolvulus	AF099104
Lucernariidae	
Haliclystus sp.	AF099103
Haliclystus sanjuanensis	AF358102
Cubozoa	
Cubomedusae	
Carybdeidae	
Carybdea marsupialis	AF358106
Carybdea rastonii	AF358108
Carybdea sivickisi	AF358110
Carybdea xaymacana	AF358109
Carukia barnesi	AF358107
Darwin carybdeid	AF358105
Chirodropidae	
Chironex fleckeri	AF358104
Chiropsalmus sp.	AF358103
Chiropsalmus quadromanus	This project
Tripedalia cystophora	L10829
Hydrozoa	
Hydroida	
Anthomedusae	
Bougainvilliidae	
Bougainvillia sp.	AF358093
Bougainvillia carolienensis	This project
Nemopsis bachii	This project
Cladonematdiae	
Cladonema californicus	AF358085
Eleutheriidae	
Staurocladia wellingtoni	AF358084
Eudendriidae	
Eudendrium racemosum	AF358094
Hydractinidae	
Podocoryne carnea	AF358092
Solanderia secunda	AJ133506
Hydridae	
Chlorohydra viridissima	AF358081
Hydra circumcincta	AF358080
Hydra littoralis	U32392
Hydra littoralis	AF358082
Moerisiidae	
Moerisia sp.	AF358083
Polyorchidae	
Polyorchis hapus	AF358089
Polyorchis penicillatus	AF358090
Scrippsia pacifica	AF358091
Porpitidae	
Porpita sp.	AF358086
Velella sp.	AF358087
Leptomedusae	
Aequoreidae	
Aequorea aequorea	AF358076
Aequorea victoria	AF358077
Blackfordiidae	
Blackfordia virginica	AF358078
Campanulariidae	
Clytia sp.	AF358074
Obelia sp.	Z86108
Laodiceidae	
Melicertissa sp.	AF358075
Tiaropsidae	
Tiaropsidium kelseyi	AF358079
Limnomedusae	
Olindiidae	
Craspedacusta sowerbyi	AF358057
Maeotias inexpectata	AF358056
Sertulariidae	

Selaginopsis cornigera	Z92899
Milleporina	
Milleporidae	
Millepora exaesa	U65484
Millepora sp.	AF358088
Siphonophora	
Calycophorae	
Diphyidae	
Muggiaea sp.	AF358073
Hippopodiidae	
Hippopodius hippopus	AF358069
Prayidae	
Nectopyramis sp.	AF358068
Praya sp.	AF358067
Sphaeronectidae	
Sphaeronectes gracilis	AF358070
Cystonectae	
Physaliidae	
Physalia physalis	AF358065
Physalia sp.	This project
Physalia utriculus	AF358066
Physonectae	
Agalmatidae	
Nanomia bijuga	AF358071
Physophoridae	
Physophora hydrostatica	AF358072
Stylasterina	
Stylasteridae	
Distichopora sp.	U65483
Trachylina	
Nacromedusae	
Aeginidae	
Aegina citrea	AF358058
Cuninidae	
Cunina frugifera	AF358059
Solmissus marchalli	AF358060
Trachymedusae	
Geryoniidae	
Liriope tetraphylla	AF358061
Halicreatidae	
Haliscera conica	AF358064
Rhopalonematidae	
Crossota rufobrunnea	AF358063
Pantachogon haeckeli	AF358062
Anthozoa	
Alcyonaria	
Alcyonacea	
Alcyoniidae	
Bellonella rigida	Z49195
Taiaroidae	
Taiaroa tauhou	AF052908
Tubiporidae	
Tubipora musica	AF052909
Gorgonacea	
Acanthogoriidae	
Acanthogorgia sp.	AF052907
Briareidae	
Briareum asbestinum	AF052912
Isididae	
Lepidisis sp.	AF052906
Primnoidae	
Narella bowersi	AF052905
Pennatulacea	
Protoptilidae	
Protoptilum sp.	AF052911
Renillidae	
Renilla reniformis	AF052581
Umbellulidae	
Umbellula sp.	AF052904
Virgulariidae	
Acanthoptilum sp.	AF052910
Ceriantipatharia	
Antipatharia	
Antipathidae	
Antipathes fiordensis	AF052900
Antipathes lata	Z92908
Bathypathes sp.	AF052901
Ceriantheopis americana	AF052898
Cerianthus borealis	AF052897
Cirripathes lutkeni	AF052902
Dendrobrachia paucispina	AF052903
Stichopathes spiessi	AF052899
Zoantharia	
Actiniaria	
Aiptasiidae	
Bartholomea annulata	This project
Nynantheae	
Actiniidae	
Anemonia sulcata	X53498
Anthopleura kurogane	Z21671
Condylactis gigantea	This project
Actinostolidae	
Stomphia sp.	AF052888
Holoclavidae	
Haloclava sp.	AF052891
Hormathiidae	
Calliactis tricolor	This project
Hormathiid anemone	AF052890
Metridiidae	
Metridium sp.	AF052889
Phymanthidae	
Epicystis crucifer	This project
Corallimorpharia	
Corallimorphidae	
Corynactis californica	AF052895
Discosmatidae	
Discosoma sp.	AF052894
Ptychodactinaria	
Ptychodactiidae	
Dactylanthus antarcticus	AF052896
Scleractinia	

Caryophyllina	
Caryophylliidae	
Ceratotrochus magnaghii	AF052886
Dendrophyllina	
Dendrophylliidae	
Enallopsammia rostrata	AF052885
Rhizopsammia minuta	Z92907
Tubastraea aurea	Z92906
Faviina	
Rhizangiidae	
Astrangia danae	This project
Phyllangia mouchezii	AF052887
Fungiina	
Agariciidae	
Pavona varians	AF052891
Fungia scutaria	AF052884
Zoanthidea	
Parazoanthidae	
Parazoanthus axinellae	U42453
Parazoanthus sp.	AF052893
Sphenopidae	
Palythoa variabilis	AF052892

Table 3. 16S rRNA sequences examined. Species are grouped taxonomically. Sequences were either obtained from GenBank (accession numbers provided) or were generated by this project (labeled as This project).

Taxa	
Scyphozoa	
Rhizostomeae	
Cassiopeidae	
Cassiopea sp.	U19374
Cassiopea xamachama	This project
Megistiidae	
Phyllorhiza punctata	This project
Rhizostomatidae	
Rhopilema verilla	This project
Semaeostomeae	
Cyanidae	
Cyanea capillata	This project
Pelagiidae	
Chrysaora quinquecirrha	This project
Ulmaridae	
Aurelia aurita	This project
Aurelia aurita	U19373
Aurelia aurita	AF461398
Aurelia limbata	AF461403
Stauromedsae	
Depastridae	
Craterolophus convolvulus	U19375
Lucernariidae	
Haliclystus sp.	U19376
Cubozoa	
Cubomedusae	
Carybdeidae	
Carybdea marsupialis	AF360118
Hydrozoa	
Hydroida	
Anthomedusae	
Bougainvilliidae	
Bougainvillia carolienensis	This project
Nemopsis bachii	This project
Clavidae	
Clava sp.	This project
Eleutheriidae	
Eleutheria dichotoma	AY169372
Staurocladia wellingtoni	AJ580934
Hydridae	
Hydra sp.	This project
Tubulariidae	
Tubularia indivisa	U19379 & this project
Leptomedusae	
Campanulariidae	
Obelia dichotoma	U19378
Siphonophora	
Cystonectae	
Physaliidae	
Physalia physalis	This project
Trachylina	
Trachymedusae	
Geryoniidae	
Liriope tetraphylla	U19377
Anthozoa	
Alcyonaria	
Alcyonacea	
Alcyoniidae	
Alcyonium sp.	U40297
Protodendron sp.	U40296
Unidentified soft coral	This project
Gorgonacea	
Holaxonia	
Acanthogoriidae	
Acanthogorgia sp.	U40301
Chrysogorgiidae	
Chrysogorgia chryseis	U40306
Gorgoniidae	
Leptogorgia virgulata	U19371
Leptogorgia chilensis	U40305
Isididae	
Acanella arbuscula	U40312
Isidella sp.	U40308
Isidid n. sp. A	U40309
Isidid n. sp. B	U40310
Lepidisis olapa	U40311
Paramuriceridae	
Paramuricea sp.	U40304
Plexauridae	
Anthomuricea sp.	U40303
Muricea fructicosa	U40302
Primnoidae	
Narella bowersi	U39786
Narella nuttingi	U40307
Scleraxonia	
Anthothelidae	
Anthothela nuttingi	U40298
Corallidae	
Corallium ducale	U40300
Corallium kishinouyei	U40313
Paragorgiidae	
Paragorgia sp.	U40299
Renillidae	
Renilla muelleri	U19372
Ceriantipatharia	
Antipatharia	

undescribed antipatharian	U40287
Antipathidae	
Stichopathes spiessi	U40286
Schizopathidae	
Stauropathes staurocrada	AY170478
Ceriantharia	
Cerianthidae	
Ceriantheopsis americana	U40289
Cerianthus borealis	U40288
Zoantharia	
Actiniaria	
Aiptasiidae	
Bartholomea annulata	This project
Edwardsiidae	
Nematostella vectensis	AY169370
Nynantheae	
Actiniidae	
Anthopleura elegantissima	AF375817
Anthopleura elegantissima	U40292
Anthopleura handi	AF375819
Anthopleura kurogane	AF375815
Anthopleura sola	AF375818
Anthopleura xanthogrammica	AF375820
Bunodosoma cavernata	AF375814
Condylactis gigantea	This project
Epiactis prolifera	AF375807
Urticina crassicornis	U91750
Urticina columbiana	U91753
Urticina coriacea	U91752
Urticina felina	U91751
Urticina lofotensis	U91754
Hormathiidae	
Hormathiid anemone	U40290
Metridiidae	
Calliactis tricolor	This project
Metridium senile	NC_000933
	This project
Phymanthidae	
Epicystis crucifer	This project
Corallimorpharia	
Actinodiscidae	
Rhodactis mussoides	AF177049
Corallimorphidae	
Corynactis californica	U40293
Scleractinia	
Astrocoeniina	
Acroporidae	
Acropora cytherea	L75995
Caryophyllina	
Caryophylliidae	
Euphyllia ancora	L76002
Euphyllia ancora	AF265598
Polycyathus muellerae	AF265606
Flabellidae	
Placotrochus laevis	AF265604
Dendrophyllina	
Dendrophylliidae	
Turbinaria peltata	L76023
Turbinaria peltata	AF265609
Faviina	
Anthemiphyllidae	
Anthemiphyllia spinifera	AF265596
Faviidae	
Cladocora caespitosa	AF265612
Favia fragum	U40295
Leptoria phrygia	L76011
Platygyra sp.	AF265611
Meandrinidae	
Dichocoenia stokesi	AF265607
Mussidae	
Cynarina sp.	AF265613
Lobophyllia hemprichii	L76013
Oculinidae	
Achrelia horrescens	L75994
Oculina patagonica	AF265601
Pectiniidae	
Mycedium sp.	AF265608
Rhizangiidae	
Astrangia danae	This project
Fungiina	
Agariciidae	
Pavona varians	L76016
Fungiidae	
Fungia fragilis	L75998
Poritidae	
Porites compressa	L76020
Zoanthidea	
Parazoanthidae	
Parazoanthus axinellae	AF398921
Sphenopidae	
Palythoa caesia	AF282931
Palythoa caribaeorum	AF282932
Protopalythoa sp.	AF398920
Zoanthidae	
Isaurus tuberculatus	AF398919
Zoanthus coppingeri	AF282936
Zoanthus coppingeri	AF282935
Zoanthus sociatus	AY049060
Zoanthus sociatus	AF282933
Zoanthus sociatus	AF282934

Table 4. Primers designed to amplify portions of the 16S rRNA in various cnidarian groups. Primer sequences are listed in a 5'-3' orientation.

Primer name	Primer sequence
General 16S rRNA	
16S_ar (Cunningham & Buss 1993)	TCGACTGTTTACCAAAAACATAGC
16S-H5' (Schroth et al. 2002)	CATAATTCAACATCGAGG
16S-H5'B	CGCAATTCAACATCGAGG
16S-H5'C	CTTAATTCAACATAGAGG
16S-H5'D	CACAATTCAACATCGAGG
Taxa-specific	
AlcyonariaF	GGACTAACGTCTAAAGCGAAACC
ScleractiniaF	GCGGTAACACTAACTGTGAA
ActiniariaF	GACCCCATTGAGCTTTACTAAAG
ActiniariaR	CATCGAGGTCGCAAACATCG
HydrozoaF	GACGAAAAGACCCTATAGAGCTTRA
HydrozoaR	CTGTTATCCCTAAGGTAGCTTTTA
ScyphozoaF	CGAAAAGACCCTATCGAGCTTT
ScyphozoaR	GGATAYCAYAATTCAACATCGAGGTYG

Table 5. Diagnostic 16S rRNA RFLPs for scyphozoan species. The fragment sizes generated by each restriction enzyme are listed for each species. DNC means that the enzyme does not cut that particular species. The area between scyphozoan primers F & R is used in this analysis. The length of the fragment amplified by these primers is listed size (bp).

Scyphozoa	Size (bp)	*Alu*I	*Ase*I	*Dpn*II	*Hpa*I
Cassiopea sp.	331	253, 78	155, 142, 34	206, 125	DNC
Phyllorhiza punctata	333	303, 18, 12	DNC	DNC	178, 155
Rhopilema verilla	335	256, 49, 18, 12	DNC	DNC	179, 156
Cyanea capillata	335	317, 18	294, 41	209, 126	DNC
Chrysaora quinquecirrha	335	317, 18	DNC	209, 126	DNC
Aurelia aurita	333	303, 18, 12	DNC	DNC	281, 52
Aurelia limbata	332	255, 47, 18, 12	DNC	DNC	DNC
Craterolophus convolvulus	335	268, 49, 18	DNC	DNC	223, 112
Haliclystus sp.	333	266, 49, 18	DNC	DNC	221, 112

Table 6. Diagnostic 16S rRNA RFLPs for hydrozoan species. The fragment sizes generated by each restriction enzyme are listed for each species. DNC means that the enzyme does not cut that particular species. The area between hydrozoan primers F & R is used in this analysis. The length of the fragment amplified by these primers is listed size (bp).

Hydrozoa	Size (bp)	*Alu*I	*Ase*I	*Dpn*II	*Nla*III	*Rsa*I
Bougainvillia carolienensis	254	216, 20, 18	223, 31	DNC	DNC	DNC
Eleutheria dichotoma	250	212, 20, 18	DNC	216, 34	DNC	146, 104
Staurocladia wellingtoni	252	214, 20, 18	128, 100, 24	DNC	DNC	DNC
Hydra sp.	252	225, 20, 18	232, 27, 4	DNC	DNC	DNC
*Tubularia indivisa (*our work)	226	188, 20, 18	129, 97	DNC	DNC	DNC
Obelia dichotoma	226	188, 20, 18	102, 93, 31	DNC	DNC	DNC
Physalia physalis	252	214, 20, 18	123, 78, 51	DNC	158, 94	DNC
Liriope tetraphylla	254	216, 20, 18	223, 31	DNC	DNC	DNC

Table 7. 16S rRNA RFLPs for anthozoan species within the orders Alcyonaria (soft corals) and Gorgonacea (sea fans). The fragment sizes generated by each restriction enzyme is listed for each species. DNC means that the enzyme does not cut that particular species. A letter designation has been assigned to each unique, gel resolvable RFLP pattern. The composite haplotype of each species is listed in parentheses underneath the taxa name and the frequency of each composite haplotype is also provided. The area between alcyonaria primer F & actinaria primer R is used in this analysis. The length of the fragment amplified by these primers is listed size (bp).

Anthozoa	Size (bp)	*Ase*I	*Dpn*II	*Hae*III
Alcyonaria				
Alcyonacea				
Alcyoniidae				
Alcyonium sp.	541	461, 80	306, 123, 112	507, 34
(AAA)		(A)	(A)	(A)
Protodendron sp.	544	464, 80	309, 235	510, 34
(ABA)		(A)	(B)	(A)
Gorgonacea				
Holaxonia				
Acanthogoriidae				
Acanthogorgia sp.	544	464, 80	309, 235	510, 34
(ABA)		(A)	(B)	(A)
Chrysogorgiidae				
Chrysogorgia chryseis	588	508, 80	DNC	554, 34
(BCB)		(B)	(C)	(B)
Gorgoniidae				
Leptogorgia chilensis	544	464, 80	309, 235	510, 34
(ABA)		(A)	(B)	(A)
Isididae				
Isidella sp.	577	597, 80	342, 235	543, 34
(CDC)		(C)	(D)	(C)
Isidid n. sp. A	561	481, 80	326, 235	527, 34
(DED)		(D)	(E)	(D)
Isidid n. sp. B	559	479, 80	324, 235	359, 166, 34
(DEF)		(D)	(E)	(F)
Lepidisis olapa	537	457, 80	302, 235	503, 34
(ABA)		(A)	(B)	(A)
Paramuriceridae				
Paramuricea sp.	544	464, 80	309, 235	510, 34
(ABA)		(A)	(B)	(A)
Plexauridae				
Anthomuricea sp.	544	464, 80	309, 235	510, 34
(ABA)		(A)	(B)	(A)
Muricea fructicosa	544	464, 80	309, 235	510, 34
(ABA)		(A)	(B)	(A)
Primnoidae				
Narella bowersi	573	DNC	338, 235	539, 34
(EFF)		(E)	(F)	(F)
Narella nuttingi	573	493, 80	338, 235	539, 34
(FFF)		(F)	(F)	(F)
Scleraxonia				
Anthothelidae				
Anthothela nuttingi	544	464, 80	309, 235	510, 34
(ABA)		(A)	(B)	(A)

Corallidae				
Corallium ducale (GGD)	562	482, 80 (G)	235, 183, 144 (G)	528, 34 (D)
Corallium kishinouyei (FFF)	572	492, 80 (F)	337, 235 (F)	538, 34 (F)
Paragorgiidae				
Paragorgia sp. (FFF)	574	494, 80 (F)	339, 235 (F)	540, 34 (F)

Haplotype	Frequency
AAA	1
ABA	3
BCB	1
CDC	1
DED	1
DEE	1
EFF	1
FFF	3
GGD	1
Total	18

Table 8. 16S rRNA RFLPs for anthozoan species within the order Actiniaria (sea anemones). The fragment sizes generated by each restriction enzyme is listed for each species. DNC means that the enzyme does not cut that particular species. A letter designation has been assigned to each unique, gel resolvable RFLP pattern. The composite haplotype of each species is listed in parentheses underneath the taxa name and the frequency of each composite haplotype is provided. The area between actiniaria primers F & R is used in this analysis. The length of the fragment amplified by these primers is listed size (bp).

Zoantharia	Size (bp)	*BamH*I	*Dpn*II	*Hpa*II
Actiniaria				
Aiptasiidae				
Bartholomea annulata	389	320, 69	235, 85, 69	DNC
(AAA)		(A)	(A)	(A)
Edwardsiidae				
Nematostella vectensis	384	314, 70	230, 84, 70	DNC
(BAA)		(B)	(A)	(A)
Nynantheae				
Actiniidae				
Anthopleura elegantissima	395	DNC	310, 85	178, 132, 85
(CBB)		(C)	(B)	(B)
Anthopleura handi	395	DNC	310, 85	178, 132, 85
(CBB)		(C)	(B)	(B)
Anthopleura kurogane	395	DNC	310, 85	217, 178
(CBC)		(C)	(B)	(C)
Anthopleura sola	395	DNC	310, 85	217, 178
(CBC)		(C)	(B)	(C)
Anthopleura xanthogrammica	395	DNC	310, 85	178, 132, 85
(CBB)		(C)	(B)	(B)
Bunodosoma cavernata	395	320, 75	235, 85, 75	217, 178
(DCC)		(D)	(C)	(C)
Condylactis gigantea	394	319, 75	235, 84, 75	177, 132, 85
(DCB)		(D)	(C)	(B)
Epiactis prolifera	395	320, 75	235, 85, 75	217, 178
(DCC)		(D)	(C)	(C)
Urticina crassicornis	415	345, 70	260, 85, 70	203, 132, 80
(EDD)		(E)	(D)	(D)
Urticina columbiana	420	345, 75	260, 85, 75	203, 132, 85
(FEE)		(F)	(E)	(E)
Urticina coriacea	420	345, 75	260, 85, 75	203, 132, 85
(FEE)		(F)	(E)	(E)
Urticina felina	415	345, 70	260, 85, 70	203, 132, 80
(EDD)		(E)	(D)	(D)
Urticina lofotensis	420	345, 75	260, 85, 75	217, 203
(FEE)		(F)	(E)	(E)
Hormathiidae				
Hormathiid anemone	389	319, 70	235, 84, 70	DNC
(GFG)		(G)	(F)	(G)
Metridiidae				
Calliactis tricolor	389	319, 70	235, 84, 70	DNC
(GFG)		(G)	(F)	(G)
Metridium senile	389	319, 70	235, 84, 70	DNC
(GFG)		(G)	(F)	(G)

Phymanthidae				
Epicystis crucifer (HGB)	394	319, 75 (H)	235, 84, 74 (G)	177, 132, 85 (B)

Haplotype	Frequency
AAA	1
BAA	1
CBB	3
CBC	2
DCC	2
DCB	1
EDD	2
FEE	2
FEF	1
GFG	3
HGB	1

Table 9. Test of the ability to amplify several species of cnidarians at a range of DNA concentrations. The amount of template DNA (in ng) is listed along with whether the amplification was successful, faint or failed. NA indicates that a given concentration was not tested. The brand of *Taq* used in the PCR is also indicated.

		Template Concentration (ng)						
Species	Taq	30	12	6	3	1	0.5	0.25
Metridium	Promega	Y	Y	Y	Y	F	F	N
	Maxx	NA	NA	Y	Y	Y	Y	Y
Epicystis	Promega	Y	Y	Y	Y	NA	NA	NA
Cassiopea	Promega	Y	Y	Y	Y	NA	NA	NA
Cyanea	Maxx	NA	NA	NA	NA	Y	Y	Y

Appendix B - List of Technical Publications

Published Technical Abstracts

B.R. Kreiser, R.L. Darden and B.D. Ortman. 2003. A preliminary report on identifying cnidarians using molecular techniques. Journal of the Mississippi Academy of Sciences 48:39.

B. R. Kreiser. Poster presentation. "Developing Molecular Methods to Identify and Quantify Ballast Water Organisms: A Test Case with Cnidarians." Partners in Environmental Technology Technical Symposium & Workshop hosted by the SERDP & ESTCP. Washington D.C., December 2-4, 2003.